Physiological responses of freshwater fish to multiple stressors in urban rivers: a transcriptomic approach

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Physiological responses of freshwater fish to multiple stressors in urban rivers: a transcriptomic approach

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Vorgelegt von

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“What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on” Jacques Yves Cousteau

To my beloved Stephanie, whose awe of the world and kind heart soothe my soul and give meaning to all

Berichterstatter/in:	PD Dr. Kathrin P. Lampert
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Contents

Abstract

This thesis investigates freshwater fish's physiological and molecular responses to multiple stressors in urban rivers, using a transcriptomic approach to understand the impacts of environmental changes, particularly salinization, on fish health. Urbanization contributes to the degradation of freshwater ecosystems, leading to altered water quality and the introduction of multiple stressors, such as salinity, temperature fluctuations, and pollution. These stressors significantly affect fish physiology, particularly in key organs like the gills and liver, which are essential for osmoregulation, respiration, and immune response. The thesis harnesses transcriptomics, a powerful tool for analyzing gene expression, to provide insights into the early molecular responses of fish to environmental stressors. Traditional methods often fail to capture the complexity of multiple simultaneous stressors, but transcriptomics allows for the detection of specific gene expression changes before they manifest in overt physiological or ecological effects. This approach is particularly valuable in understanding how fish maintain homeostasis, compensate for environmental challenges, and potentially recover from stress, offering a detailed view of species' responses to urban-induced environmental changes.

The thesis is organized into three chapters, each addressing different aspects of how freshwater fish respond to multiple anthropogenic stressors. The first chapter examines the impact of varying chloride concentrations on gene expression related to osmoregulation in *Gasterosteus aculeatus*. The second chapter explores tissue-specific molecular responses to extreme salinity in invasive hybrid minnows, providing insights into their physiological resilience and potential invasiveness. The third chapter investigates the combined effects of multiple stressors, including temperature and dissolved oxygen variations, on gene expression in *Cottus rhenanus*. The findings consistently highlight salinity as a critical factor influencing fish health, even when other stressors are present. This research underscores the ecological threat posed by freshwater salinization and the importance of incorporating transcriptomic data into conservation strategies. The thesis contributes to a growing understanding of how urbanization and environmental change impact freshwater ecosystems and provides valuable molecular biomarkers for assessing the health of fish populations in increasingly urbanized environments.

Future research directions include integrating transcriptomics with epigenetics to provide a more comprehensive understanding of how multiple stressors shape physiological responses and adaptability in organisms. This integrative approach can enhance conservation efforts by offering early indicators of stress and informing strategies to protect freshwater biodiversity in the face of global human-driven change.

General introduction

1. Background and rationale

1.1 Urbanization: Impacts on freshwater ecosystems

Urbanization is projected to expand significantly, with the United Nations Population Division (UNPD) estimating that urban land cover could increase by 1.2 to 1.8 million km² by 2030 (UNPD, 2019). This rapid urban expansion presents severe challenges to freshwater ecosystems, contributing to a phenomenon commonly known as "Urban Stream Syndrome." This syndrome encompasses a range of detrimental effects on streams, including altered channel morphology, highly variable hydrographs, reduced biotic richness, and elevated levels of nutrients and contaminants (McDonald et al., 2019; Walsh et al., 2019).

Moreover, urban streams are increasingly subjected to nonpoint source pollution and climate variability, which introduce complex "chemical cocktails" into aquatic environments, functioning as multiple stressors (Kaushal et al., 2018; Schäfer et al., 2023). These stressors are major threats to freshwater biodiversity and ecosystem stability, contributing to the decline of riverine biodiversity and altering community structures (Barrett et al., 2022). In this context, fluctuations in water quality, driven by multiple anthropogenic stressors such as pH, salinity, temperature, dissolved oxygen, and pollution, emerge as significant factors influencing the distribution and health of fish species (Bernhardt et al., 2020; Menon et al., 2023).

1.2 The impact of multiple stressors on freshwater fish

Water quality is a critical factor influencing fish distribution and habitat suitability. Key parameters such as temperature, conductivity, dissolved oxygen, and salinity all play significant roles. Conductivity, which measures ion concentrations like $HCO3^-$, $SO42^-$, and Cl^-, tends to be elevated in disturbed catchments, including urban areas where pollution is prevalent (Kefford et al., 2023). Hypoxia, or low oxygen levels, is a global water pollution issue that exacerbates sublethal effects in fish, including endocrine disruption and oxidative stress (Abdel-Tawwab et al., 2019; Pollock et al., 2007; Zhu et al., 2013). As ectotherms, fish are particularly susceptible to temperature fluctuations. Chronic increases in temperature can compromise their ability to manage other stressors, leading to significant ecological consequences (Alfonso et al., 2021).

Freshwater salinization, caused by urban runoff and other anthropogenic activities, further threatens fish populations by increasing stress levels and mortality rates, which in turn affects biodiversity and ecosystem functionality (Cunillera-Montcusí et al., 2022). Fish must expend considerable energy on osmoregulation in higher salinity conditions, which impacts their growth, development, and respiration. This often leads them to avoid high-salinity areas, further disrupting their distribution (Guh et al., 2015; Tseng & Hwang, 2008). Additionally, the prevalence of micropollutants—chemicals entering surface waters from wastewater effluents, untreated wastewater, urban runoff, and agricultural runoff—poses significant risks to aquatic ecosystems. These pollutants can be absorbed by fish, causing notable physiological responses and contributing to ecosystem degradation (Kidd et al., 2024; Kumar et al., 2020).

Fish gills and the liver are crucial organs for oxygen uptake, osmoregulation, temperature regulation, detoxification, and immune response. These organs are highly sensitive to environmental stressors and serve as key indicators of physiological stress (Escobar-Sierra et al., 2024; Escobar-Sierra & Lampert, 2024a, 2024b; Evans et al., 1999; Jeffries et al., 2021). The presence of multiple stressors in urban rivers complicates the assessment of their individual and combined effects. The complex interactions among these stressors make it challenging to evaluate their direct impact on fish physiological responses in real-world conditions (Orr et al., 2024).

2. The role of transcriptomics in environmental biology and research gaps

2.1. An overview of transcriptomics in environmental biology

Transcriptomics, the comprehensive analysis of RNA transcripts to assess gene expression across the entire genome, has revolutionized environmental monitoring by providing deep insights into the physiological responses of organisms to environmental stressors. Traditional methods for evaluating the impact of environmental changes on aquatic organisms, such as endpoint mortality studies, laboratory manipulations, and population-level analyses, often fall short of capturing the full complexity of how multiple, simultaneous stressors affect organisms in their natural habitats (Jeffries et al., 2021; Semeniuk et al., 2022). Transcriptomics provides a sensitive measure of physiological performance, identifying specific molecular pathways and physiological processes affected by stressors (Lowe et al., 2017; Wikelski & Cooke, 2006).

This method is particularly valuable for environmental biology because it enables researchers to detect early molecular responses to stress before they manifest in visible physiological changes or mortality. By analyzing changes in gene expression, transcriptomics can identify

key physiological thresholds, allowing researchers to model species responses to a range of environmental conditions and stress gradients (Connon et al., 2018). This capability is crucial for understanding how organisms maintain homeostasis, compensate for environmental challenges, and recover from stress. For instance, Somero et al. (2016) highlighted the importance of distinguishing between compensatory mechanisms that allow organisms to survive sublethal stress and those responses that lead to irreversible damage. This approach extends traditional ecological and physiological measures, offering a detailed understanding of species responses to environmental stressors.

Furthermore, transcriptomics can enhance both hypothesis-driven research and exploratory studies by providing pathway-specific insights that go beyond traditional molecular biomarkers. This approach enables the development of molecular and biochemical biomarker suites that are tailored to specific functional pathways, offering more precise assessments of environmental impact (Lowe et al., 2017). As a result, transcriptomics not only complements but also extends the capabilities of traditional ecological and physiological measures, providing a more nuanced understanding of species responses to environmental stressors.

2.2. Transcriptomics in fish conservation physiology and management

Transcriptomic studies have increasingly revealed the intricate impacts of environmental stressors on fish, uncovering how gene expression changes in response to various stress gradients. As pointed by Connon et al. (2018) such studies can identify critical inflexion points in gene expression, delineating ranges of physiological homeostasis, compensation, and potential recovery, as well as predicting disease states or mortality (Figure 1). By integrating transcriptomic data with conventional physiological and ecological metrics, researchers can bridge molecular mechanisms with ecological outcomes, providing a holistic view of species responses.

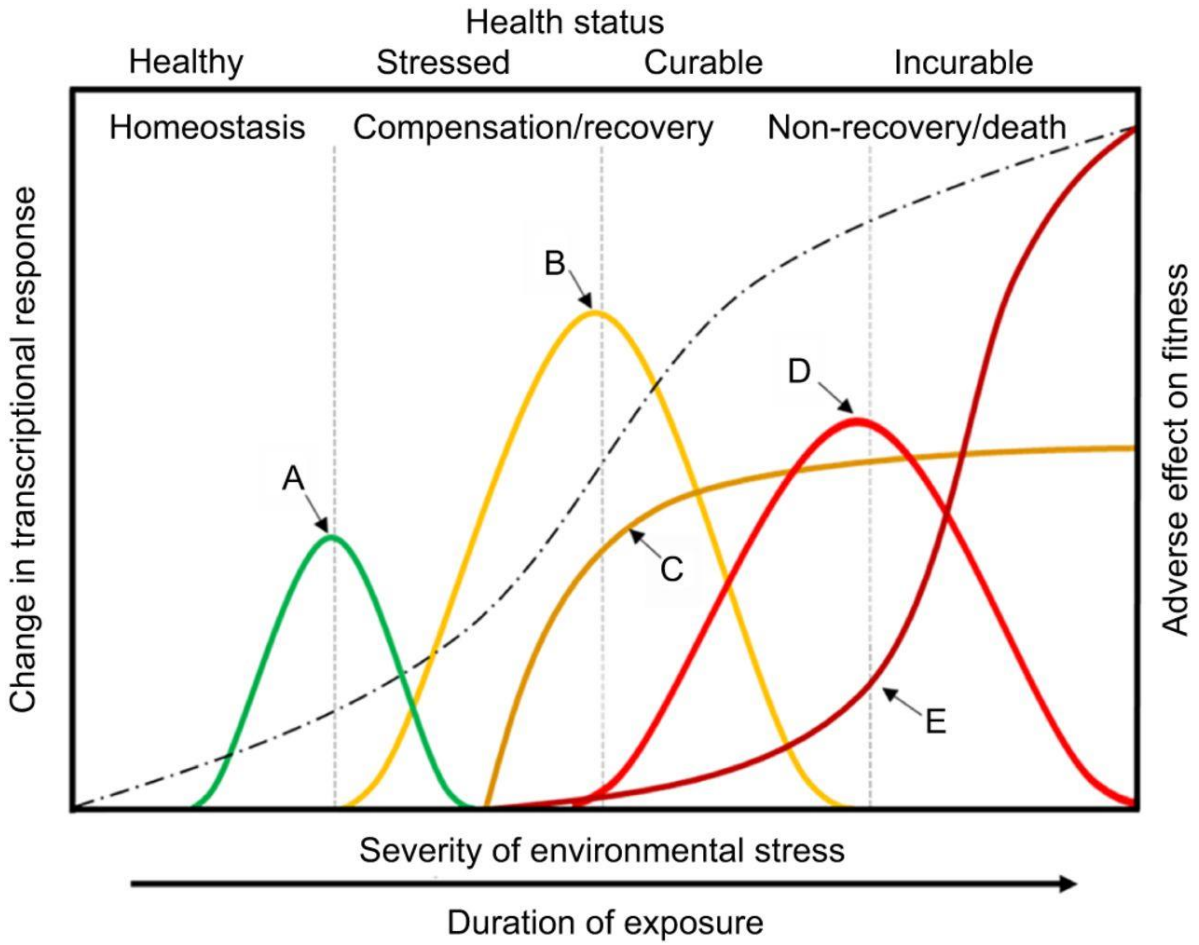


Figure 1. Conceptual transcriptional response patterns across various functional pathways as environmental stress intensifies or exposure time increases. Points of inflexion may signal particular adverse effects and functional limits, such as homeostasis and compensation (A), possible reversibility and recovery (B), or predictions of disease and potential mortality (C, D, E). The dashed black line illustrates a hypothetical sigmoidal relationship between adverse effects on fitness (right-hand y-axis) and the severity of environmental stress (Connon et al., 2018).

Significant research has demonstrated the value of transcriptomics in assessing the effects of environmental stressors on fish. For example, transcriptomic analyses have been used to elucidate the molecular mechanisms underlying salinity tolerance in fish species. Studies have identified key genes and pathways involved in osmoregulation, immune response, and stress adaptation, revealing the genetic basis of tolerance mechanisms (Chen et al., 2021; Guo et al., 2018; Vij et al., 2020). These insights have potential applications in conservation and management, as they highlight targets for improving fish resilience to salinity changes. Research has also explored how transcriptomics can detect subtle physiological changes that may not be evident through traditional measures. For instance, transcriptomic profiling has helped identify specific molecular pathways affected by stressors such as temperature fluctuations, salinity changes, dissolved oxygen and pollution (Escobar-Sierra et al., 2024; Escobar-Sierra & Lampert, 2024a, 2024b; Jeffrey et al., 2023; Komoroske et al., 2016). These

studies have expanded our understanding of how fish cope with environmental stressors and have provided new biomarkers for assessing stress responses. Despite these advancements, there are notable gaps in the current research. Much of the existing transcriptomic research has been conducted under controlled laboratory conditions, often using stressor levels that exceed those typically encountered in natural environments (Whitehead & Crawford, 2006). This limitation restricts the applicability of findings to real-world scenarios where stressor levels and interactions are more variable and complex.

Field studies are essential to address these gaps, as they allow for the observation of gene expression patterns in response to a broader range of environmental stressors. Natural environments present fluctuating and unpredictable challenges that can offer unique insights into how fish adapt to real-world conditions (Rojas-Hernandez et al., 2019). Field-based transcriptomic studies can provide a more accurate representation of how environmental changes impact fish health and behaviour, leading to more effective conservation strategies. Additionally, most transcriptomic studies have focused on a limited number of model species and tissues, particularly gills, which are critical for respiration and osmoregulation but represent only one aspect of fish physiology (Lin et al., 2020; Zhou et al., 2020). Expanding research to include multiple tissues and non-model species would offer a more comprehensive understanding of how various stressors affect overall fish physiology. For instance, analyzing liver tissue could reveal insights into detoxification processes and metabolic responses, while investigating other non-model species could provide broader perspectives on stress adaptation and resilience.

Furthermore, there is a need for de novo transcriptomic studies that generate foundational data for species not yet studied extensively. These studies can provide crucial insights into baseline gene expression profiles and stress responses, which are essential for developing tailored conservation strategies. As more transcriptome profiles are archived and meta-analytical tools are developed, the ability to elucidate specific functional response pathways will improve, enhancing our understanding of the mechanisms regulating responses to environmental changes (Semeniuk et al., 2022). Translating these findings into actionable conservation strategies will require effective communication and collaboration between researchers, stakeholders, and regulatory agencies. By addressing these gaps and leveraging transcriptomic data, researchers can better inform management practices and conservation efforts aimed at protecting freshwater biodiversity in increasingly urbanized environments.

Aims

The overarching aim of this thesis is to investigate the physiological and molecular responses of freshwater fish to multiple stressors in urban rivers, with a focus on understanding the impact of salinization and other anthropogenic factors. Using transcriptomic analysis to assess the molecular and physiological responses of freshwater fish to various environmental stressors in urban rivers. This approach combines field sampling of fish from different sites and conditions with advanced gene expression profiling techniques to identify key genes and pathways involved in stress responses. Each chapter addresses specific research questions to elucidate these impacts.

Chapter 1 seeks to determine how varying chloride concentrations along a salinity gradient influence gene expression related to osmoregulation in *Gasterosteus aculeatus*.

Chapter 2 examines the tissue-specific molecular responses of *Phoxinus septimaniae* x *P. dragarum* to extreme salinity levels caused by potash mining, aiming to understand how these responses affect the fish's physiological resilience.

Chapter 3 explores how multiple anthropogenic stressors, including variations in temperature, salinity, and oxygen levels, impact gene expression and physiological responses in *Cottus rhenanus* across different seasons and sites in an urban river system.

Through these investigations, the thesis aims to provide a comprehensive understanding of how urban-induced stressors affect freshwater fish at both molecular and physiological levels.

Summary of chapters

To address the central research questions, this thesis is organized into three chapters, each focusing on specific aspects of how freshwater fish respond to multiple stressors in urban rivers.

Chapter 1: Field Application of De Novo Transcriptomic Analysis to Evaluate the Effects of Sublethal Freshwater Salinization on *Gasterosteus aculeatus* in Urban Streams

Chapter 1 investigates how varying chloride concentrations along a salinity gradient affect gene expression related to osmoregulation in *Gasterosteus aculeatus*. By sampling fish from different salinity levels in the Boye River and conducting de novo transcriptomic analysis, the study identifies significant differential gene expression associated with salinity stress. The findings reveal key genes and pathways involved in osmoregulation, highlighting the activation of ion transport mechanisms in response to sublethal salinity changes. These results underscore the need to reconsider current salinity thresholds and demonstrate the utility of transcriptomic approaches for monitoring freshwater ecosystems impacted by salinization.

Chapter 2: Unravelling the Molecular Mechanisms of Fish Physiological Response to Freshwater Salinization: A Comparative Multi-Tissue Transcriptomic Study in a River Polluted by Potash Mining

Chapter 2 examines the tissue-specific molecular responses of *Phoxinus septimaniae* x *P. dragarum* to extreme salinity levels resulting from potash mining in the Llobregat River. The study focuses on gene expression in the brain, gills, and liver to understand how these tissues adapt to high salinity conditions. The analysis reveals distinct molecular responses in each tissue, with a notable impact on osmoregulation, metabolic processes, and immune functions. The findings provide insights into the physiological resilience of invasive fish species under severe salinity stress and highlight the importance of managing potash mining pollution to protect native fish populations and aquatic biodiversity.

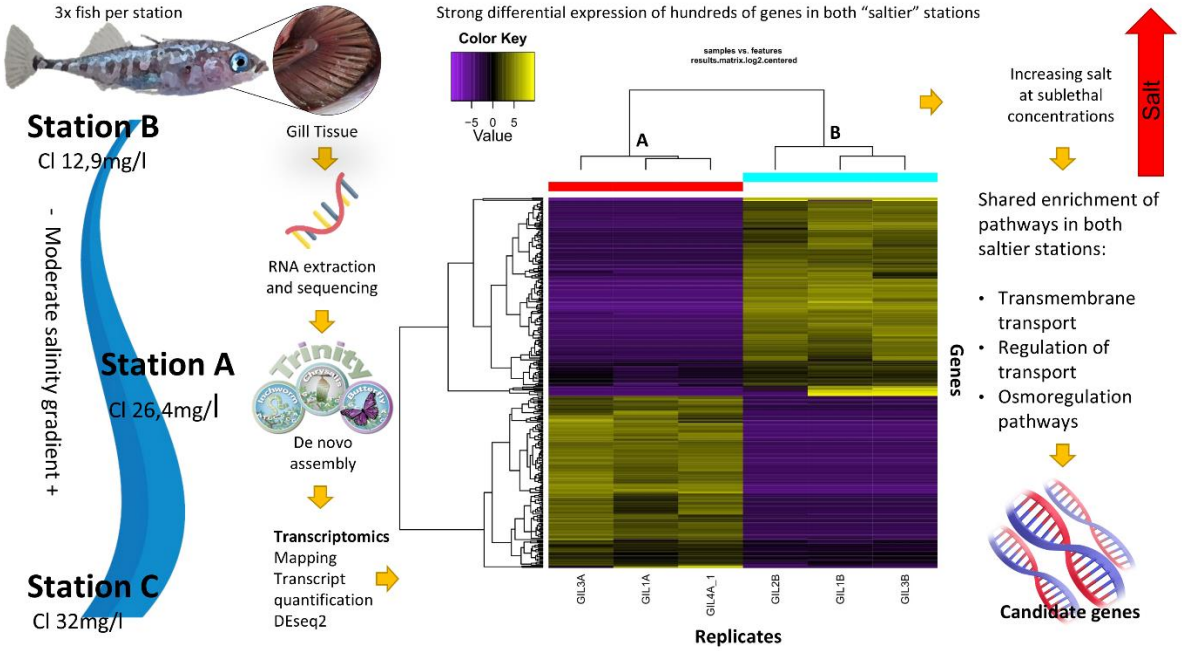
Chapter 3: Navigating the Urban River: Transcriptomic Responses of Freshwater Fish to Multiple Anthropogenic Stressors

Chapter 3 explores how multiple anthropogenic stressors, including variations in temperature, salinity, and oxygen levels, affect gene expression and physiological responses in *Cottus rhenanus* in the Emscher River. The study employs transcriptomic analysis to assess how these

stressors influence gene expression related to metabolism, osmoregulation, oxidative stress, and immune responses across different seasons and sites. The results reveal significant seasonal and site-specific variations in gene expression, emphasizing the complex interactions between multiple stressors and their cumulative impact on fish health. This research underscores the importance of integrating transcriptomic data into conservation strategies to mitigate the effects of urban-induced stressors on freshwater species.

Chapter 1: Field application of de novo transcriptomic analysis to evaluate the effects of sublethal freshwater salinization on *Gasterosteus aculeatus* in urban streams

Graphical abstract:



Keywords: Salinity; Transcriptome analysis; Gene ontologies; Gene expression; Fish physiology; Chlorides; Ion transport.

Highlights:

- Sublethal chloride activates costly osmoregulatory systems.
- Differential expression of osmoregulation-related genes observed.
- Ion transport genes key in salinity stress adaptation.
- Transcriptomics reveal transmembrane transport genes' response.
- Study highlights freshwater salinization's molecular impact on fish.

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RESEARCH ARTICLE

Field application of de novo transcriptomic analysis to evaluate the effects of sublethal freshwater salinization on *Gasterosteus aculeatus* in urban streams

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Abstract

Freshwater salinization poses global challenges for aquatic organisms inhabiting urban streams, impacting their physiology and ecology. However, current salinization research predominantly focuses on mortality endpoints in limited model species, overlooking the sublethal effects on a broader spectrum of organisms and the exploration of adaptive mechanisms and pathways under natural field conditions. To address these gaps, we conducted high-throughput sequencing transcriptomic analysis on the gill tissue of the euryhaline fish *Gasterosteus aculeatus*, investigating its molecular response to salinity stress in the highly urbanized river Boye, Germany. We found that in stream sections with sublethal concentrations of chloride costly osmoregulatory systems were activated, evidenced by the differential expression of genes related to osmoregulation. Our enrichment analysis revealed differentially expressed genes (DEGs) related to transmembrane transport and regulation of transport and other osmoregulation pathways, which aligns with the crucial role of these pathways in maintaining biological homeostasis. Notably, we identified candidate genes involved in increased osmoregulatory activity under salinity stress, including those responsible for moving ions across membranes: ion channels, ion pumps, and ion transporters. Particularly, genes from the solute carrier family SLC, aquaporin *AQP1*, chloride channel *CLC7*, ATP-binding cassette transporter *ABCE1*, and ATPases member ATAD2 exhibited prominent differential expression. These findings provide insights into the potential molecular mechanisms underlying the adaptive response of euryhaline fish to salinity stress and have implications for their conservation and management in the face of freshwater salinization.

Introduction

Urbanization, as reported by the United Nations Population Division (UNPD), is poised to expand urban land cover by 1.2–1.8 million Km^2 by 2030 [1]. The negative impacts of this global urban expansion on freshwater habitats and biodiversity have led to the coining of the term "Urban Stream Syndrome" to describe the myriad impacts on streams, including altered

collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

channel morphology, highly variable hydrographs, diminished biotic richness, and elevated concentrations of nutrients and contaminants [2–4]. One particularly striking consequence of urbanization is the accumulation of various ions, such as Na^+, Ca^{2+}, Mg^{2+}, K+, NO_3^-, SO_4^{2-}, Br, and Cl^-, contributing to freshwater salinization—an increasingly pressing issue drawing attention from researchers and policymakers [5–8]. A variety of factors can lead to heightened levels of ion accumulations, including background concentrations in watersheds, as well as human-driven changes in land use, sea level rise, agriculture, road salts, wastewater, and mining [8]. Given this background, the Ruhrgebiet region in Western Germany offers an excellent case study for examining freshwater salinization in urban streams. This area is one of the most densely populated in Europe [9] and encompasses a wide variety of activities that are known to contribute to a higher vulnerability of freshwater systems to salinization. Mining is one of the major threats in this region, with coal extraction requiring the infiltration of groundwater that is subsequently pumped into nearby rivers and streams [10]. As a result, mining water discharge has been recorded to reach chloride concentrations as high as 3500 mg/L in the River Lippe which is 175 times higher than the typical natural background chloride concentrations in the world surface freshwater ecosystems of <20 mg/L [11]. However, the implementation of new management measures and the closure of many mines has led to a significant reduction in concentration, with current levels now below 400 mg/L [10]. Despite this progress, the ongoing inputs from abandoned coal mines in Western Germany continue to contribute to freshwater salinization in the region [12]. Another significant factor contributing to freshwater salinization in the Ruhrgebiet region is the changing land cover. As urbanization has intensified, rivers and wetlands have become increasingly surrounded by impervious man-made surfaces. This has led to a greater runoff of road salts and sewage waters, particularly in urban surface waters which turns them particularly vulnerable to salinization [13].

Most freshwater animals are strictly adapted to low ion concentrations in their surrounding water compared to their internal ion concentrations (stenohaline), and spend a lot of energy for osmoregulation by actively pumping ions inside their bodies and excreting water [14]. Thus, hyperosmotic stress due to freshwater salinization poses a significant physiological challenge to many freshwater animals, including fish which must maintain their ion homeostasis in the face of ion loss through diffusion and water absorption across their permeable membranes [15]. Active membrane transport of ions against concentration gradients is required to maintain internal salt concentrations, and increased environmental salinity above the internal ion concentrations leads to greater energy expenditure for osmoregulation [16]. As a result of the advancement in fish osmoregulation physiology research, and their importance in bioindication, fisheries, and the economy, fish have become essential models for studying the effects of environmental change [17]. Chloride is widely recognized as the most important anion for osmoregulation in freshwater, making it one of the most commonly used indicators to measure salinity effects on fish [18]. The annual upper chloride mean threshold concentrations in German surface waters, as per the European water directive, range between 40–90 mg/L, with Canada and the U.S. having chronic thresholds of 160 mg/L and 230 mg/L, respectively [11, 19]. However, these thresholds have been based on ecotoxicological tests on a limited number of species that often rely on measuring the salinity effect on endpoint survival and may overlook sublethal effects on non-model species. In ecotoxicology, sublethal effects are defined as those that do not directly cause the death of an individual but rather have an effect on individual fitness (i.e. behavior, cognition, physiology) [20, 21]. This is critical, as it is recognized that one of the major challenges of understanding the impact of environmental change is determining the point at which sublethal responses to stressors begin to adversely impact the organism [22]. Focusing solely on tissue, organ, or endpoint mortality effects and using a limited number of model species may overlook critical information regarding the

physiological impacts of sublethal salinity stress on organisms. Moreover, until recently, studies on fish physiology have relied on technologies with reduced resolution and a limited range of species models [18]. Thus, there is a pressing need to apply novel technologies and widen the model species pool to gain a better understanding of the effects of salinity stress on freshwater species.

In this context, the application of molecular techniques has greatly improved our understanding of the toxicity mechanisms of major anthropogenic ions in various fish species at high resolution. This development aligns with the recent scientific consensus on the analysis of the response of fish to environmental stressors at different levels, including molecular gene expression and potential adaptation [23]. Furthermore, the molecular adaptation to salinity by freshwater species has been identified as one of the major gaps in the research agenda of global freshwater salinization, and transcriptomic technologies have been proposed as a promising approach to tackle it [6]. Transcriptomics allow capturing a snapshot in time of the total gene expression (mRNA) present in a cell under a given condition [24]. And as noted by Connon et al., (2018) [25], can reveal sublethal stress thresholds that extend beyond the negative impacts on individual and population fitness. This provides a more comprehensive understanding of species' habitat requirements, which in turn facilitates their management. Recently, transcriptomics has been applied to identify the function of novel and conserved genes and the underlying pathways of salinity stress in fish species such as *Lateolabrax maculatus*, *Poecilia reticulate*, *Pseudopleuronectes yokohamae*, *Lates calcarifer*, *Acipenser baeri*, *Oreochromis mossambicus* female × *O. urolepis hornorum* male, *Oncorhynchus keta*, and *Gasterosteus aculeatus* [26–33]. While the studies previously cited have provided insight into the mechanisms underlying tolerance thresholds and adaptations to osmotic stress, they have been limited by their reliance on laboratory manipulations and exposure to salinity concentrations that exceed sublethal thresholds. As highlighted by Whitehead & Crawford (2006) [34], it is crucial to investigate expression variation in ecological contexts to fully comprehend the role of gene expression in the adaptive response to environmental stressors. Under natural conditions, organisms are exposed to a variety of fluctuating environmental signals, providing a valuable opportunity to discern gene expression patterns that are not discernible under laboratory conditions [35].

The threespine stickleback (*Gasterosteus aculeatus*) is an opportunistic species that has demonstrated an exceptional capacity for adaptation to diverse aquatic habitats and is widely distributed in the Ruhrgebiet region in Western Germany. One key trait that has enabled this species to thrive in various environments and colonize systems throughout the Northern Hemisphere is its ability to regulate its osmotic balance in response to changes in salinity (Euryhalinity) [36]. Even landlocked freshwater populations of stickleback, which have been separated from the marine environment for thousands of years, possess the osmoregulatory machinery needed to handle abrupt changes in salinity [37]. This suggests that sticklebacks have maintained their physiological adaptation to saltwater despite repeated colonization of freshwater habitats. Their wide distribution and adaptability have made them an important model in the study of adaptive evolution and have been subject to the development of large-scale genetic and genomic resources [38]. Furthermore, experimental evidence has shown that their gill transcriptome responds to subtle changes in environmental salinities within the freshwater range [28]. Although their euryhalinity suggests they can tolerate the salinity changes, it has been noted that the activation of the hyperosmotic osmoregulatory mechanisms comes at a high energetical cost [39]. Cellular remodeling, enzyme expression, and transport protein synthesis linked to salinity changes have been estimated to account for 20–68% of the total energy expenditure in certain fish species [40]. These costs may drive the need for physiological and behavioral optimization to minimize energy expenditure, resulting in narrow

physiological tolerance windows and behavioral avoidance of salinities outside the optimal range [39, 41]. However, there is still a lack of knowledge on the molecular mechanisms underlying this species' responses to salinity stress. Therefore, the threespine stickleback provides an excellent model for studying the mechanisms underlying the osmoregulation of a euryhaline opportunistic species under sublethal salinity concentrations.

Taking into consideration the research gaps and opportunities introduced above, we designed a study with the objective of understanding the physiological effects on *G. aculeatus* under subtle changes in concentrations of chloride from anthropogenic sources in the field. We are shifting our focus from controlled manipulative studies to the application of transcriptomics in environmental field research. Specifically, we aim to understand how the salinity gradient in the urban stream affects the gene expression profile in the gills of *G. aculeatus*, with a particular focus on pathways related to osmoregulation. We hypothesize that the salinity gradient in the urban stream will affect the gene expression profile of *G. aculeatus* gills, with an enrichment of osmoregulation-related pathways. To test this hypothesis, we sampled fish from an urban stream in North Western Germany with a historical salinization influence at three stations along a salinity gradient. We then evaluated the transcriptomic fingerprint of *G. aculeatus* gills, performed a comparative transcriptomic study, evaluated the gene ontology of the differentially expressed genes and identified candidate genes related to osmoregulation. Ultimately, our goal was to utilize de novo transcriptomic analysis to gain insights into the impact of sublethal freshwater salinization on this euryhaline species in urban streams. This research not only aims to uncover molecular adaptation mechanisms but also to contribute in broadening the range of model species used in the study of sublethal effects of salinity stress on freshwater organisms.

Material and methods

Sampling design

We choose three stations in a gradient of chloride concentration in two tributaries and in the main channel of the river Boye (Fig 1). The station with the highest salinity of the stations is in the Kirchschemmsbach, a stream that has historically affectation by salinity intrusion (Station C). Followed by the main channel of the Boye in its confluence with the Kirchschemmsbach (Station A). Station B is a station in a stream close to the other stations but with no historical or present influence of secondary salinization. It was therefore used ascontrol site. The chloride concentrations in the period of sampling for station B were 12.9 mg/L, Station A 26.4 mg/L, and station C 32.0 mg/L. In the sites that we sampled, the concentration was below the annual chloride mean upper threshold from German streams in the central highlands which is in the range of 40–50 mg/L [12]. And below the reported chronic or acute toxicity level for species such as trout and fathead minnow [42]. However, both stations A and C are above the typical Cl^- concentrations from natural sources in surface freshwater ecosystems that are in general below 20 mg/L [11].

Gasterosteus aculeatus (Linnaeus) individuals were sampled using electrofishing equipment. Fishing and electrofishing permits were given by the Untere Fischereibehörde Kreis Recklinghausen (Genehmigung_Recklinghausen_20210824) and the Emschergenossenschaft (Genehmigung_EGLV_Entnahme_20210901114640736). Fish were then quickly euthanized using an overdose of tricaine methanesulfonate (MS222) before harvesting the gill tissue, immediately fixing it in RNAprotect (QIAGEN), and storing it at -20˚C. In total nine samples were obtained, three from the control (Station B), three from the mid-salinity station (Station A), and three from the high-salinity station (Station C).

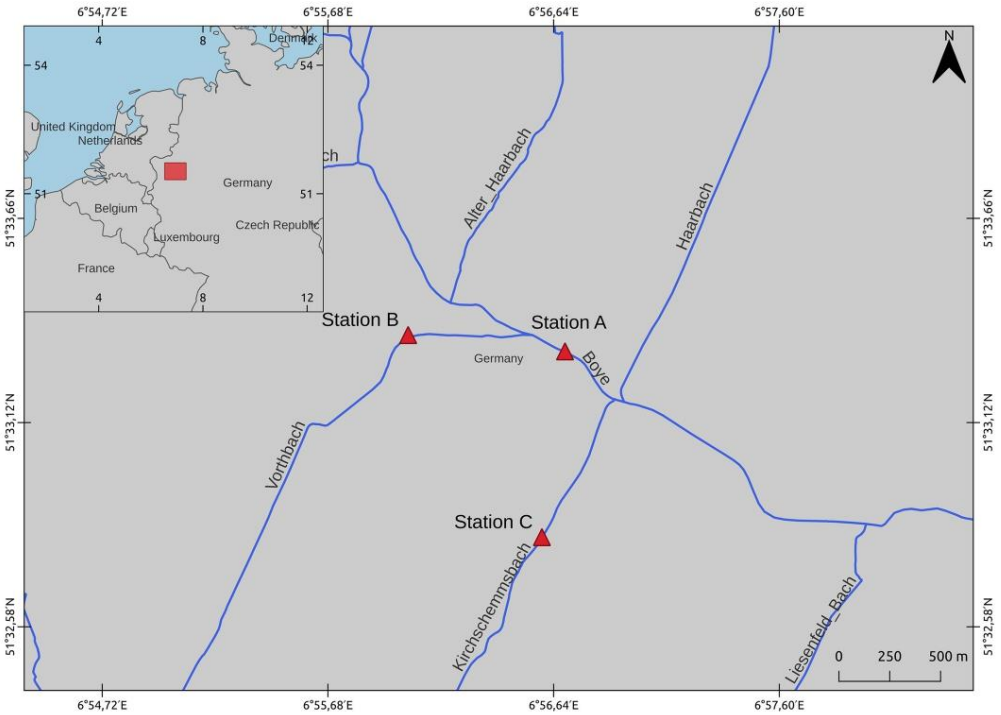

Fig 1. Location of the sampling station in the Boye catchment. The Chloride concentrations in the period of sampling for the upstream station B (Control) are 12.912 mg/L. And the two downstream treatment stations: Station A 26.378 mg/L, and station C 32 mg/L.

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RNA isolation and Illumina sequencing

The fixed tissue of each sample was homogenized in a solution of buffer 700μl RLT with a 10μl:1ml concentration of β-mercaptoethanol in a FastPrep-24™ (MP Biomedicals) bead beater during 30s at 5 m/s in a screw cap vial with 1mm diameter glass beads. Afterwards, the RNA was extracted using a RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. The quality of the extractions was checked using a Nanodrop 1000 Spectrophotometer (Peqlab Biotechnologie, Germany), ensuring that the concentration was >50ng/μl, and that OD 260/280 = 1.8–2.1, and OD 260/280>1.5. Next, the RIN^e value quality was assessed using the RNA ScreenTape system in an Agilent 2200 TapeStation, ensuring that the samples had a RIN^e score over 7.0. An Illumina Tru-Seq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) was applied in generating sequencing libraries following the manufacturer's protocol. After purifying, Illumina sequencing was carried out on an Illumina/Hiseq-2500 platform (Illumina, San Diego, CA, USA) to generate 100 bp paired-end reads with 50 million reads depth by the Cologne Center for Genomics (CCG), Germany. The choice of read depth followed CCG technical advice, which estimated that given a published genome size of approximately 200,000 genes, this read depth would be adequate for constructing a de novo transcriptome with nine biological replicates.

Sequence quality control, de novo assembly, and annotation

Raw sequences of all nine samples were checked in FASTQC [43] for quality, followed by a quality improvement via an adaptor and low-quality reads filtering using TRIMMOMATIC, with a threshold of phred + 33 quality score of at least 25 for all bases, and a minimum length of 50 bp for all reads kept for downstream analyses [44]. TRINITY v2.9.1 [45] was used to create a de novo assembly using the nine pairs of clean sequences. The reads of the raw nine paired sequences were aligned to the de novo transcriptome using SALMON [46] as the abundance estimation method. BUSCO v5.2.2 [47] was used to assess transcriptome assembly completeness by searching against the actinopterygii_odb10 (Creation date: 2021-02-19) dataset. The expression matrix was generated and the low expressions transcripts (minimum expression of 1.0) were filtered using TRINITY v2.9.1, the expression levels were normalized as transcripts per million transcripts (TPM). Identification of likely protein-coding regions in transcripts was performed with TRANSDECODER v5.5.0 [48]. The filtered transcriptome sequencing reads were aligned to protein, signal peptides, and transmembrane domains using the tools, DIAMOND v2.0.8 [49], SIGNALP 6.0 [50], TMHMM v2.0 [51], and, HMMER v3.3.2 [52]. The de novo transcriptome was then functionally annotated using the tool TRINOTATE v3.2.2 [45].

Differential gene expression analysis

Before the differential gene expression analysis, the SALMON tool was used to align the clean reads of the three sites to the filtered transcriptome and the mapping rate was calculated. The mapping tables were merged according to the stations and using TMM [53] normalization. Differential expression analysis with three biological replicates of the two stations with higher chloride (A and C) were compared to the reference condition (Station B) using the DESeq2 [54] package, the fold change cut-off was set at >2, and the FDR$\leq$0.05. This analysis assesses thousands of gene expression differences between the two stations and the control. Therefore, the incorporation of the false discovery rate (FDR) within DESeq2 is essential for mitigating type I errors, making it a widely accepted standard in genomics for correcting false positive results in differential expression analysis [55, 56]. A heatmap plot using GGPLOT2 [57] was used to represent the differentially expressed genes (DEGs) between each treatment and the control site. The significant upregulated and downregulated DEGs ontology terms were annotated and a gene ontology (GO) pathway enrichment analysis was performed using ShinyGo [58] (version 0.61). An enrichment analysis finds the GO terms with unbalanced distribution between two groups of genes or probe sets. To account for multiple testing issues, a FDR correction was performed on the P-values of this comparison to control falsely rejected hypotheses [59]. The genes identified in the GO analysis to be related to the pathways involved in osmoregulation were extracted and listed. The assembly was performed in the High-performance computing system of the university of Cologne (CHEOPS) and the rest of the analysis was performed using the Galaxy project platform [60].

RNA-seq qPCR validation

To validate the results from the RNA-seq analysis, a subset of eight osmoregulation-related upregulated genes was chosen and assessed via real-time quantitative PCR analysis (qPCR). The primer design was performed using the Primer-Blast software [61], and all primer sequences are listed in Table 1. Firstly, reverse transcription of the total RNA samples was performed using the ReverAid first-strand cDNA synthesis kit (Thermo Scientific), and the resulting cDNA was diluted 10-fold and used as a template for qPCR. The qPCR was performed using PowerUp™ SYBR™ Green Master Mix in a StepOne Real-Time PCR system following the

Table 1. Primer sequences used for differentially expressed genes used for qPCR validation.

Genes	Forward primer	Reverse primer
PTPN3	GCTGATTTGGGCTACGGTGC	GTGTGTACGGCCATTCTGCG
ANO10	AACACGGGGACAGTGAGGAG	CCGAACGGACGCCTACAAGA
ANO9	CGCCTCCACCTTTGTTTCCG	CATCGGTGAGGAGGAGGCAA
AQP1	CGGTCGCTGGAGCTCTGTAA	GGAAACGGGAGCACCAGACT
NEDD4	GTGTGGGTCGTAGCCACTGT	TGATGTCGAGCAGCCTGGAT
PTPN6	TGTGCAGACAAGCGTAAGCC	CTGCAGCGTAGTGGGGAAGT
SH3BP2	GTCTTCTTTCGGTCCAGCCG	GTGTCGGCGCTGTTCTCCAT
SLC9B2	GAGCCTGTGGAGAAGGCGAT	GTGGTCCCCTCCATGTTGCT

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manufacturer's indications. The qPCR was carried out in triplicate for each sample with a final well volume of 20μl. An initial 10-minute hot start at 95˚C, followed by 45 cycles of 30 seconds of denaturation at 95˚C and 1-minute annealing at 59˚C. Followed by a melting curve ranging from 60˚C to 95˚C with data acquisition every 0.3˚C. Each gene was assessed using the $2^{-\triangle\triangle Ct}$ method, with *GADPH* as the housekeeping gene for normalization, the optimized sequence for the gene was obtained from [37]. Finally, the sample with the lower expression of the control station was used as the reference to compare the expression.

Results

Sequence quality control, de novo assembly, and annotation

The RNAseq was performed on the library products from the gill of *G. aculeatus*, resulting in an average of 47,945,147, 64,032,899.33, and 68,881,883.67 total reads from stations A, B and C respectively. The quality score %Q30, which states the sum of the reads with a base call accuracy of 99, 9% was 99.32%, 99.36% and 99.37% respectively. After quality control, an average of 44,571,717, 59,441,980.67 and 64,633,936 were retained for stations A, B and C respectively. All the retained reads had >Q30. The assembly with all sequences produced a raw transcriptome of 559.3mb, with a N50 of 3797bp, with 3319 complete BUSCO's and a completeness of 91.2%. The transcriptome was filtered for low-expression transcripts, and from a total 340418 in the raw transcriptome, 98643 were retained (28.98%). The filtered transcriptome outcome was 182.4mb of sequences, with a N50 of 3735bp, 2334 complete BUSCO's and a completeness of 64.1%. In total 50155 transcripts were annotated which represents 50.8% of the de novo transcriptome.

Differential gene expression analysis

The mapping rate of the individual sequences to the filtered transcriptome was an average of 60.68%, 59.78% and 60.18% on stations A, B and C respectively. When comparing the station with mid-salinity (A) with the reference site (B), a total of 627 genes (306 down- and 321-regulated) (Fig 2). For the comparison of the station with high-salinity (C) with the reference site (B), a total of 611 genes (270 down- and 341regulated) (Fig 3). In both heatmaps the pattern of expression was similar in all three sample replicates per station. The significant DEGs were assigned to three major Gene Ontology (GO) categories: biological process, cellular component, and molecular function. The DEGs were classified in a total of 200 and 193 higher-level GO terms for A and C, respectively, relative to the reference station (S1 File). Our comparative analysis of the high-level GO terms within the significant DEG dataset, identified potential osmoregulation-related terms, including transporter activity (GO:0005215), transmembrane transporter activity (GO:0022857), and channel regulator activity (GO:0016247).

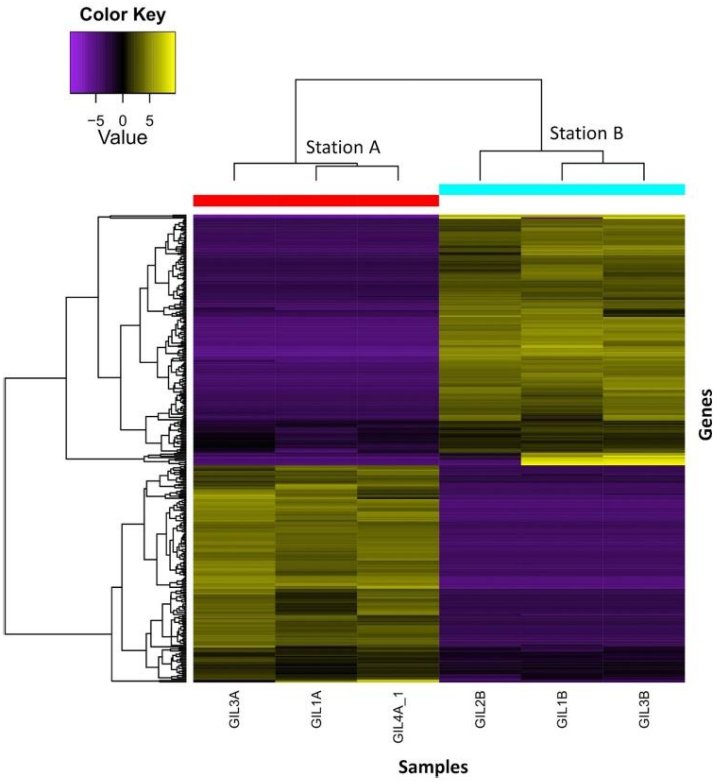

Fig 2. Heatmap displaying 627 differentially expressed genes between the mid-salinity station (A) and the reference station (B). The X-axis represents sample replicates per station, and the Y-axis represents individual gene expression. Upregulated genes are shown in yellow, with brighter colors indicating higher expression values. In contrast, purple shades indicate downregulated genes, with the brightest shade indicating the strongest downregulation.

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The GO ontology analysis was used to find which terms were enriched in the two higher salinity stations compared with the reference site for the upregulated and downregulated genes separately. Furthermore, to identify the candidate DEGs related to these particular osmoregulation-related GO terms the gene codes were extracted and assigned to the correspondent functional category term (S2 File). For the upregulated genes we found a set of enriched GO terms for the three categories, molecular function, cellular component and biological process (Fig 4). The main enriched GO terms related to osmoregulation in the upregulated DEGs in the biological process GO category for station A vs B are: Regulation of sodium ion transmembrane transport (GO:1902305 and GO:1902306), regulation of sodium ion transmembrane transporter activity (GO:2000649 and GO:2000650), and regulation of sodium ion transport (GO:0002028 and GO:0010766). For the molecular function the GO categories main enriched terms were related with the activation and regulation of sodium channels (GO:0017080,

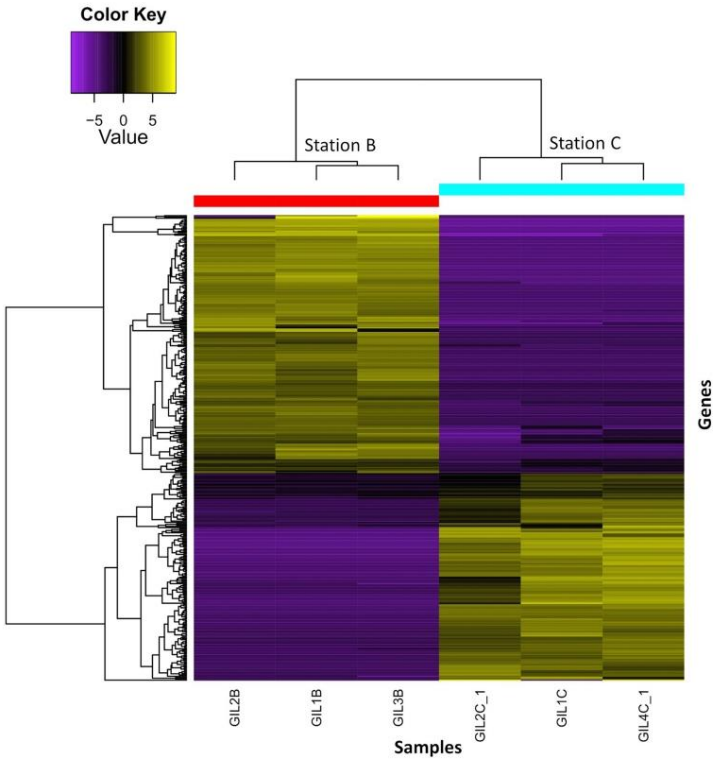

Fig 3. Heatmap displaying 611 differentially expressed genes between the high-salinity station (A) and the reference station (B). The X-axis represents sample replicates per station, and the Y-axis represents individual gene expression. Upregulated genes are shown in yellow, with brighter colors indicating higher expression values. In contrast, purple shades indicate downregulated genes, with the brightest shade indicating the strongest downregulation.

https://doi.org/10.1371/journal.pone.0298213.g003

GO:0019871, GO:0016248, GO:0016247). The main enriched GO terms related to osmoregulation in the upregulated DEGs in the biological process GO category for station C vs B are: Regulation of sodium ion transport (GO:0002028), regulation of ion transmembrane transporter activity (GO:0032412), regulation of transmembrane transporter activity (GO:0022898), regulation of transporter activity (GO:0032409), regulation of ion transmembrane transport (GO:0034765) and sodium ion transmembrane transport (GO:0035725). For the molecular function GO category in C vs B the osmoregulation-related enriched term was: Sodium channel regulator activity (GO:0017080). For the cellular component category only the osmoregulation-related term transport vesicle (GO:0030133) was found to be enriched.

For the downregulated genes we found a set of enriched GO terms for the three categories, molecular function, cellular component and biological process in the A vs B and only in the molecular function and biological process terms for C vs B (Fig 5). For station A vs B, we

Fig 4. Gene ontology analysis of enriched upregulated genes for stations A and C compared to the reference site B. The number of genes is displayed on the X axis, while the Y axis represents GO categories colour-coded based on molecular function (red), cellular component (green), and biological process (blue). Could you unify the X axis to make a comparison easier? (max value AB = 16, max value CB = 18).

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found enriched GO terms related to osmoregulation only in the molecular function GO category: Anion binding (GO:0043168) and ATP binding (GO:0005524). The main enriched GO terms related to osmoregulation in the downregulated DEGs are in the molecular function GO category for station C vs B and are mostly related to the Ras, Rho and Rab small GTPases terms. These related terms are: Ras guanyl-nucleotide exchange factor activity (GO:0005085), Regulation of Ras protein signal transduction (GO:0046578), Rho guanyl-nucleotide exchange factor activity (GO:0005085), Rho GTPase binding (GO:0017048), GTPase regulator activity (GO:0030695), GTPase activator activity (GO:0005096) and Rho GTPase binding (GO:0031267).

In summary we found enriched GO terms specifically associated with osmoregulation in both upregulated and downregulated genes within the higher salinity stations compared to the reference site.

From the enriched GO terms that were identified to be related to osmoregulation for both higher salinity stations, we extracted all the DEGs and listed them in S3 File. Then we performed a literature search of all the extracted genes and listed the DEGs with referenced

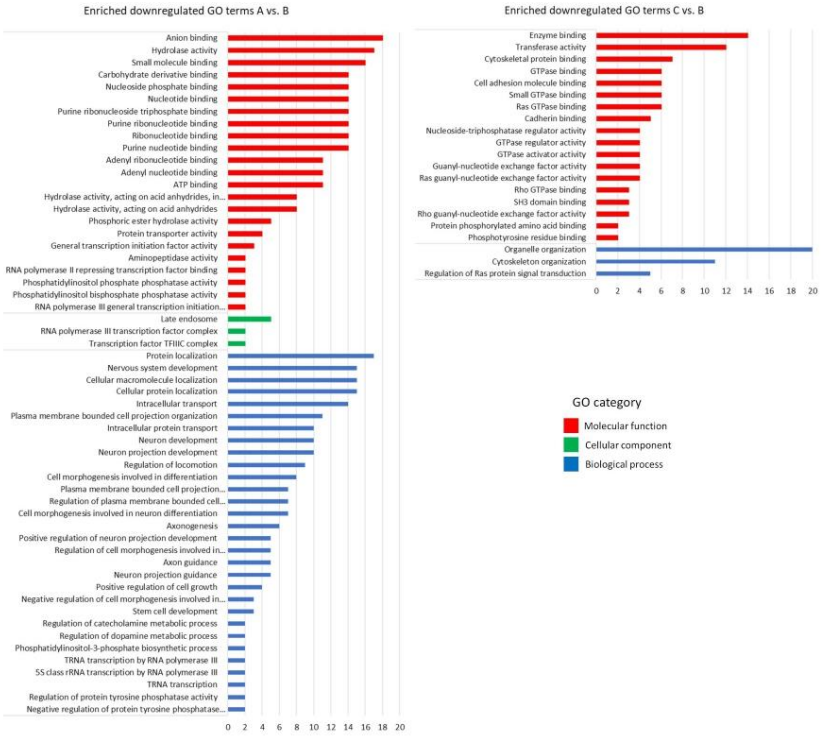

Fig 5. Gene ontology analysis of enriched downregulated genes for stations A and C compared to the reference site B. The number of genes is displayed on the X axis, while the Y axis represents GO categories colour-coded based on molecular function (red), cellular component (green), and biological process (blue). X axes–see above.

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osmoregulation involvement (Table 2). For station C vs B, the genes with the higher upregulation were the protein transporters *SEC31A*, followed by *SEC24B*. Then, the regulators of the ion channel and ion transporters *PTPN3*, *PTPN6*, *AHCYL1* and *NEDD4* were also found to be upregulated. In the last position were other genes involved in ion transport, the cation channel protein anoctamin 9 *ANO9* and the solute carrier *SLC9B2*. The strongest downregulated genes in C vs B, were the Ras, Rho and Rab small GTPases related genes *ITPKB* and *AKAP13*. And finally, the genes involved in ion transport, the ion channel *TRPM7* and the solute carrier *SLC9B2*. For station A vs B, the genes with the higher upregulation were the regulators of ion channel and ion transporters *PTPN3*, *NEDD4* and *NEDD4L*. Followed closely by the channel protein-encoding gene *AQP1* and the solute carrier *SLC35B2*. In the last position with lower expression were the channel regulators *PRF1*, *CLCN7*, the solute carrier *SLC6A15*, and the channel protein Anoctamin 10 *ANO10*. The strongest downregulation was recorded for the membrane tension regulator *SCARB2*, the transporter *ABCE1*, the channel regulators *VPS35* and *VP26C* and the protein complex gene *PIKFYVE*. In the last position was the anion exchanger *ATAD2*.

Table 2. List of the selected candidate genes involved in osmoregulation generated from the Gene Ontology analysis. Upregulated genes are highlighted in red, while downregulated genes are highlighted in blue, with intensity indicating expression level (Log2 FC). The false discovery rate (FDR) value is also included, indicating that an FDR $p<0.05$ suggests a non-random differential expression of the gene.

Group	Gene	Description	Log_2 FC	FDR(p)
Station C vs. Station B	*SEC31A*	Protein transport protein Sec31A, ABP125, ABP130, SEC31-like protein 1, SEC31-related protein A, Web1-like protein	12.35	6.54E-17
	SEC24B	Protein transport protein Sec24B, SEC24-related protein B	11.71	4.12E-16
	PTPN3	Protein tyrosine phosphatase non-receptor type 3	11.17	2.79E-11
	AHCYL1	Adenosylhomocysteinase like 1	10.57	8.07E-06
	NEDD4	Nedd4 e3 ubiquitin protein ligase	10.06	3.00E-09
	PTPN6	Protein tyrosine phosphatase non-receptor type 6	10.00	7.48E-14
	ANO9	Anoctamin 9	9.47	1.63E-07
	SLC9B2	Solute carrier family 9 member b2	9.15	6.22E-08
	TRPM7	Transient receptor potential cation channel subfamily M member 7	-2.74	4.53E-03
	SLCO5A1	Solute carrier organic anion transporter family member 5A1	-9.05	1.07E-10
	AKAP13	A-kinase anchor protein 13, AKAP-13Non-oncogenic Rho GTPase-specific GTP exchange factor	-12.53	3.02E-18
	ITPKB	Inositol-trisphosphate 3-kinase B, 2.7.1.127, Inositol 1,4,5-trisphosphate 3-kinase B, IP3 3-kinase B, IP3K B, InsP 3-kinase B	-13.11	3.00E-24
Station A vs. Station B	*PTPN3*	Protein tyrosine phosphatase non-receptor type 3	11.22	1.35E-17
	NEDD4	Nedd4 e3 ubiquitin protein ligase	10.87	7.59E-06
	NEDD4L	Nedd4 like e3 ubiquitin protein ligase	10.31	9.20E-11
	AQP1	Aquaporin 1 (colton blood group)	8.09	5.37E-07
	SLC35B2	Solute carrier family 35 member b2	7.55	0.01
	PRF1	Perforin 1	3.65	0.01
	CLCN7	Chloride voltage-gated channel 7	2.48	6.49E-05
	SLC6A15	Solute carrier family 6 member 15	2.40	0.01
	ANO10	Anoctamin 10	2.23	0.01
	ATAD2	ATPase family AAA domain-containing protein 2, 3.6.1.-	-2.19	0.0002
	VP26C	Vacuolar protein sorting-associated protein 26C	-8.67	2.3E-04
	PIKFYVE	1-phosphatidylinositol 3-phosphate 5-kinase, Phosphatidylinositol 3-phosphate 5-kinase, 2.7.1.150	-10.39	3.76E-11
	VPS35	Vacuolar protein sorting-associated protein 35	-10.52	2.24E-13
	ABCE1	ATP-binding cassette sub-family E member 1, 2'-5'-oligoadenylate-binding protein	-11.50	4.86E-11
	SCARB2	Lysosome membrane protein 2	-12.67	3.7E-20

In summary, both of the salinity sites showed enrichment of pathways related to osmoregulation. Furthermore, we extracted and listed candidate genes related to the pathways of osmoregulation.

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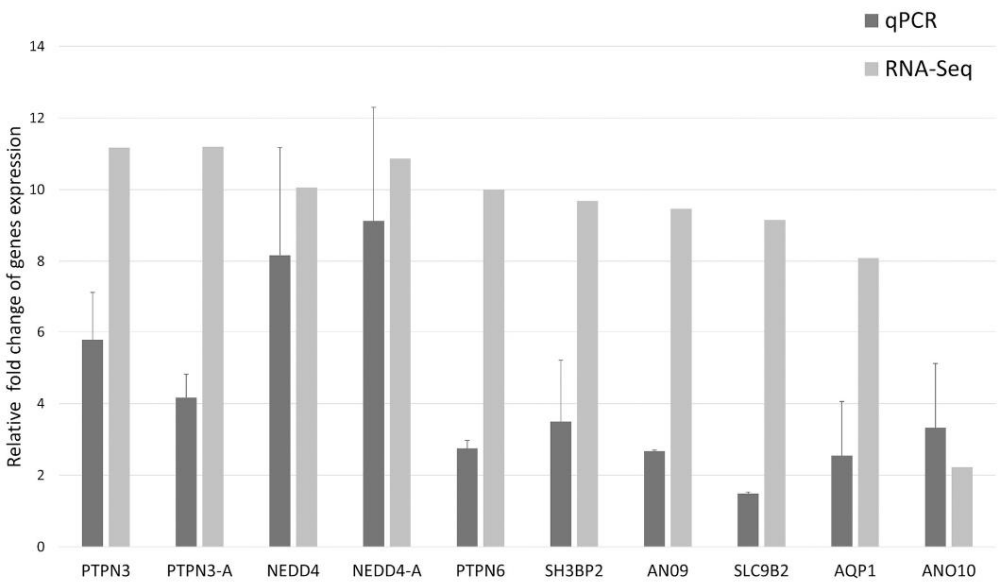

Fig 6. qPCR validation plot of RNA-seq data. It shows the expression of eight genes, including *NEDD4* and *PTPN3*, in two treatment groups. *NEDD4* and *PTPN3* refers to group C vs. B, while *NEDD4*-A and *PTPN3*-A refer to group A vs. C. The gene names are on the X axis, and the Y axis displays the relative fold change in gene expression (Log2FCs). Mean ± standard deviation Log2FC values are presented.

https://doi.org/10.1371/journal.pone.0298213.g006

RNA-seq data validation by qPCR

The analysis of the melting curve of the qPCR resulted in a single product for all genes. The visual comparison of the relative fold changes of the subset of eight DEGs showed a consistently positive direction in both qPCR and RNA-Seq analysis (Fig 6), which suggests that the RNA-seq analysis is reliable.

Discussion

Our de novo transcriptome assembly yielded over 50 million reads with 100% of reads surpassing Q30, and a N50 length of 3735bp, indicating high-quality raw data. The mapping rate of the individual sequences to the transcriptome, which was consistent with previous studies [29, 62], was close to 60% for all stations, demonstrating the effectiveness and reliability of the high-throughput sequencing transcriptomic analysis. Additionally, the maintained trend in gene expression between qRT-PCR and RNA-seq validated our results and confirms their suitability for further analysis. The results of the quality control mechanisms validate our sampling design, demonstrating that a depth coverage of 50 million reads and three biological replicates per treatment suffice for the differential gene expression analysis using a de novo transcriptome approach. According to our prediction, we found a differential expression of the genes in the gill tissue of *G. aculeatus* under a gradient of salinity in the field. Notably, even when chloride concentrations were well below reported toxic thresholds, our comparative transcriptomics approach was able to evidence the activation of the energetically costly osmoregulatory system. Furthermore, we were able to identify candidate genes related to increased

osmoregulatory activity even for subtle changes of chloride concentration. In the ontology analysis, we found an enrichment of DEGs genes predominantly related to transmembrane transport and regulation of transport. It is to highlight, that in neither of the treatments we found DEGs with functional categories that could be related to the handling or euthanasia of the fish. The pathways and genes that we found fall in one or more of the big three classes of proteins in charge of moving ions across membranes, ion channels, ion pumps and ion transporters which are essential for all living organisms [63]. These key groups of proteins maintain the osmotic pressure in exchange for considerable quantities of energy, and together receive the name "Transportome" [64]. The transportome is in charge of maintaining biological homeostasis through a balance between the intracellular and extracellular environments and currently accounts for 10% of the protein-coding human genome [65]. For instance, studies with Chinese mitten crab, Guppy *Poecilia reticulata*, *Acipenser baeri*, hybrid tilapia, and *G. aculeatus* showed a significant number of DEGs involved in ion transport and transmembrane transport in varying saline habitats from estuaries to marine environment [26, 28, 29, 32, 66]. These results confirm the importance of the transportome in the adaptive response to salinity changes and align with the enrichment of genes in related categories in our study.

The enrichment of proteins related to gill tissue transportome in response to salinity changes in our study aligns with consistent findings across various transcriptomic investigations, spanning from invertebrates to fish, as elaborated below. Malik and Kim (2021) identified that about 40% (162/405) of the DEGs were classified as belonging to the transportome in a meta-analysis of four different salinity challenge studies in Chinese mitten crab. In a study with the Guppy *Poecilia reticulata* it was also shown that a large number of DEGs were involved in ion transport and transmembrane transport [26]. Similarly, when hybrid tilapia underwent various laboratory salinity stress treatments,, numerous ion transport enzymes and ion transporters associated with osmoregulation were identified [32]. Another example, involves the Siberian sturgeon (*Acipenser baeri*), where gill transcriptome was sequenced after a high salinity stress exposure. Several pathways were enriched under salinity stress, and as in our case there was a set of DEGs related to osmoregulation like genes of the CLC family and Small GTPases, together with other ion transport mechanisms when challenged by salinity [29]. In additional comparative gill transcriptomic studies, the euryhaline fishes *Acanthogobius ommaturus*, *Lates calcarifer* and *Lateolabrax maculatus* were exposed to a salinity gradient manipulation. Strikingly, their GO analysis of the gill transcriptome responded similar to ours, with an enrichment of ion transport, cell volume regulation and signaling pathways [33, 62, 67]. While we found only one example of comparative gill transcriptomics involving *G. aculeatus*, the study revealed differential expression of several genes related to 'transmembrane transport' when exposed to varying salinities [28]. Even when all the previously cited studies converge with our findings in the enrichment of osmoregulatory, cellular signaling and transmembrane ion transport in the gill transcriptomic of fish when facing salinity stress. It is noteworthy that that our findings add to the previous knowledge by demonstrating this osmoregulatory pathway enrichment in wild-caught fish under a natural gradient of salinity stress.

In the following sections, we discuss the relevance of the enriched pathways for osmoregulation, the specific function of the candidate genes, and the ecological implications of our findings for euryhaline fish such as *G. aculeatus* in the face of global freshwater salinization.

Membrane transporters DEGs

Membrane transporters were the most abundant group of candidate genes that were differentially expressed in the stations with higher chloride concentrations. The candidate genes

related to these pathways are *VPS35*, *VPS26*, *SLC35B2*, *SLC9B2*, *SLCO5A1*, *SLC6A1*, *SEC31A*, and *SEC24*. The Vacuolar protein sorting-associated proteins *VPS35* and *VPS26* are components of the cargo recognition sub-complex of the retromer, and together they recognize and bind to specific sorting signals on cargo proteins to be transported back to the Golgi apparatus [68, 69]. Both proteins are crucial in cell homeostasis, by regulating the transport of particles and by controlling the water reabsorption protein Aquaporin-3 (Aquaporin-3) [70]. According to the review by Verri et al., (2012) [71], the *SLCs* transporters in teleost fish, are conformed by over 50 families with 380 known members and facilitate the transportation of nearly all soluble molecules across cellular membranes. *SLC35B2* is part of the nucleoside-sugar transporter family (*SLC35*) that is involved in the endoplasmic reticulum and Golgi of eukaryotic cells [72]. Similar to our findings, differential expression of *SLC35* family members was reported in the gills of the Chinese mitten crab in response to changes in salinity [66]. A remarkable finding was the upregulation of *SLC9B2*, as the members of the *SLC9* family play a critical role in regulating pH in cells and organelles, as well as maintaining acid-base and volume homeostasis [73]. In previous studies a high upregulation of *SLC9* genes has been recorded under salinity stress, in particular, *SLC9B2* was found to function as a Na^+/H^+ exchanger in an experiment using Xenopus oocytes [74]. *SLCO5A1* from the *SLC5* Sodium-glucose cotransporter family and *SLC6A15* from the SLC6 Sodium- and chloride-dependent neurotransmitter transporter family were also differentially expressed in our study and are also important transporters found in fish [71]. Furthermore, The SLCs have shown to hold high importance in generating counter-ion fluxes to maintain ion homeostasis while avoiding overtly high membrane tension [75]. In addition to observing an enrichment of SLC proteins under salinity stress, we also found an increase in proteins related to the vesicular traffic of the SEC family, such as *SEC31A* as it was also found in Malik and Kim (2021), and *SEC24B*. The impairment of the expression by glucocorticoids of members of the *SEC24* family has been related to the aberrant ion and macromolecular transport in Zebrafish [76]. Overall, the findings suggest that these candidate genes play an important role in maintaining ion and macromolecular transport under salinity stress.

Ion channel regulation and transport DEGs

Another important group of DEGs enriched terms was the one related to ion channel regulation and transport. Examples of this that we pinpointed as candidate genes are *PTPN3*, *PTPN6*, *NEDD4*, *NEDD4L*, *AHCYL1*, *AQP1*, *TRPM7*, *PRF1*, *CLCN7*, *ANO9* and *ANO10*. This holds particular importance as ion channels such as voltage-gated, ligand-gated, and second messenger-gated channels, nonselective cation channels, and epithelial Na^+ and Cl^- channels are key to maintaining cellular homeostasis [77]. And as described in Davis et al., (2001) [78] protein tyrosine phosphatases as *PTPN3* and *PTPN6* are involved in tyrosine phosphorylation, which is the primary means of regulating the majority of voltage-gated, ligand-gated, and second messenger-gated channels. *PTPN3* has been found to regulate the high-osmolarity glycerol (HOG) *MAPK* pathway, which is essential for yeast survival in a hyperosmotic environment [79]. Other important regulators that we propose as candidate genes are *NEDD4* and *NEDD4L*, which have been found to mediate ubiquitination and degradation of *AQP2* which is a key protein in water homeostasis [80]. Also, the *AHCYL1* protein has been identified as a regulator of the intracellular Ca^{2+} channel inositol 1,4,5-trisphosphate (IP3), and multiple ion channel and ion transporters [63]. *AQP1* is a member of the aquaporin gene family, a group of channel proteins that are involved in the transport and other solutes in the presence of an osmotic gradient. *AQP1* differential expression has been previously detected in fish gill, kidney and gut tissue in response to salinity stress for various species [81, 82]. *TRPM7* is a

plasma-membrane protein expressed ubiquitously, which belongs to the melastatin-related transient receptor-potential ion channel *TRPM* subfamily and possesses both ion channel and α-kinase domains. It has been observed that *TRPM7* is involved in channel regulation via changes in intracellular Ca^{2+} concentration when subject to osmolality gradients [83]. The perforin (*PRF1*) was also differentially expressed, which is relevant as it is known to be able to polymerize and form channels in cell membranes of target cells [84]. Also relevant was the differential expression of the chloride channel (*CLC7*), which was also found to be expressed under osmotic stress in the gills of tilapia under osmotic stress [32]. Finally, we found upregulation of both transmembrane proteins anoctamin 9 and 10 (*ANO9* and *ANO10*). Anoctamins are a family of Ca^{2+}-activated Cl^- channels and phospholipid scramblases that have been shown to support cell volume regulation [85, 86]. The differential expression of these genes highlights their potential role in cellular homeostasis regulation under osmoregulatory stress conditions. Further studies on these candidate genes could lead to a better understanding of the adaptive mechanisms used by fish to cope with environmental stressors.

Small GTPases, anion and ATP binding DEGs

Another set of pathways that were found to be differentially expressed are those related to the Ras, Rho and Rab small GTPases, anion and ATP binding genes. The Ras, Rho and Rab small GTPases proteins are key regulator and signaling mediators of a wide variety of process that occur in eukaryotic cells [87]. The main importance of these proteins in the context of our study lies in their direct interaction with ion channels to regulate ion homeostasis [88]. Furthermore, Ras GTPases are involved in the process of membrane thinning and curvature with effects on osmoregulation [89]. One important candidate gene found in our study was the Rho GTPase *AKAP13*, which is a key regulator of ion homeostasis under osmotic stress. In detail, *AKAP13* attracts *JIP4* and activates *NFAT5* through the Rho-type small G-proteins and p38 *MAPK* signalling pathway, regulating intracellular osmolarity [90]. *ITPKB* regulates the subcellular distribution of Rasa3, a Ras GTPase-activating protein and has been observed at is differentially expressed when hyper-osmotically stressed [91, 92]. In the gene ontology categories, anion and ATP binding we highlight the candidate genes *ABCE1*, *SCARB2*, *PIKFYVE* and *ATAD2*. *ABCE1* is a member of the ABC gene family, which constitutes the largest group of transmembrane transporter proteins encoded in the human genome. These proteins utilize ATP as an energy source to facilitate the transportation of various molecules across cellular membranes keeping cell homeostasis [93]. The lysosomal integral membrane protein 2 (*SCARB2*) is a transporter of cholesterol that is suspected to control membrane tension and ion trafficking via the direct insertion of lipids [94]. *PIKFYVE* is the sole source of all the Phosphoinositide lipids PI(5)P pool in yeast membranes and is a key regulator of ion transport, and it is upregulated under hyperosmotic shock in yeast [95]. Finally, another interesting candidate is *ATAD2*, a gene from the ATPase family. Its relevance resides in the fact that ATPases are enzymes that utilize ATP to power many cellular processes including transmembrane transport and osmoregulation in fish [96, 97]. Note that the differential expression of *ABCE1* and *ATAD2* provides direct evidence of the energetic cost of osmoregulation, even at low salinity concentrations in our study. The proposed candidate genes play important roles in transmembrane transport, ion trafficking, and osmoregulation.

Ecological implications

Due to the inherent complexities of our field study design, where several uncontrolled factors may influence the gene expression of *G. aculeatus*, we are cautious in establishing a direct causal relationship between the differential gene expression and the salinity gradient of the

stations. Nonetheless, our results strongly suggest that the gill osmoregulatory mechanism of *G. aculeatus* is activated at sublethal concentrations of chloride, and we have identified candidate genes that could serve as indicators of salinity stress. Using a transcriptomic approach, we detected activation of osmoregulatory pathways in both study sites, even below the thresholds established by German authorities for running waters. This finding is significant, as transcriptomics has been successful in determining the sublethal effects of various stressors and giving new insights into the management of species and ecosystems. For example, Komoroske et al., (2016) [39], were able to determine that the endangered delta smelt (*Hypomesus transpacificus*) osmoregulatory system was activated at a high energetically cost under sublethal salinity thresholds. And for the same species, again using transcriptomic coupled with physiological methods, it was demonstrated that sublethal critical effects are observed 4–6˚C below the previously established acute tolerance limits [98]. The relevance of this transcriptomic study was further demonstrated when Brown et al., (2016) [99], combined the information on these sublethal thresholds to model the future habitat suitability of this species. Based on these precedents, we predict that our results have the potential to inform models on the expanding distribution beyond its native range of *G. aculeatus* in response to freshwater salinization. *G. acualeatus* populations have been increasing rapidly in several ecosystems around the world in response to altered conditions, with important negative impacts on native species that have led to consider it an invasive species in places such as lake Constance (Germany) [100, 101]. An effect that has been previously described for other invasive species that have demonstrated adaptive advantage in increasing salinity scenarios, increasing their populations and their distributions [102, 103].

It is worth noting that the candidate genes related to osmoregulation discussed in our study, which showed differential expression, have primarily been identified in manipulative experiments involving significant changes in salinity. This highlights the relevance of our findings, as we have identified the same candidate genes within the subtle salinity gradient of our field experiment, suggesting their potential as biomarkers for rapidly assessing sublethal salinity stress in field applications. Furthermore, we were able to measure a physiological response to a stressor in a wild non-model organisms in field conditions where many other stressors may be at interplay. Therefore, our study outlines the potential of transcriptomic approaches for assessing physiological responses of wild organisms in conditions with known or unknown stressors and be tools for informing management and policy decisions [22, 25, 104]. As our understanding of the mechanisms of osmoregulation advances with the use of transcriptomics, more biomarkers or candidate genes are identified. The expression of these selected biomarkers can then be used in transcriptomic arrays to evaluate the responses of individual fish to several important biological and environmental stressors such as water-quality stressors, aquatic invasive species, and climate change [105]. Thus, further studies into the mechanisms and candidate genes that we present here for sublethal salinity stress have the potential to have an impact on the conservation and management of freshwater ecosystems in the prospect of increasing freshwater salinization. In the light of our findings, we suggest to perform further studies into other non-model wild-caught fish physiological stress response using a combination of techniques that include transcriptomics. We consider that by including these, the current thresholds for salinity and other pollutants in freshwater could be revisited as sublethal effects would be accounted for. Our study has shown that even small changes in salinity significantly change gene expression patterns in fish. Enhanced salinity could pose energetic costs to fish thereby reducing long term survival and reproductive output. This emphasizes the critical need to preserve the highest water quality in streams, recognizing that sublethal stressors can exert profound impacts on ecosystems.

Conclusion

In conclusion, our transcriptomics analysis of the gill tissue of *G. aculeatus* under a gradient of salinity in the field strongly suggests a significant differential expression of genes related to membrane transport and regulation of transport. Our results support the notion that the transportome plays a crucial role in the adaptive response to salinity changes in euryhaline fish and highlight the activation of energetically costly osmoregulatory systems even when chloride concentrations are below the established toxic thresholds. We would like to highlight our finding of a set of candidate genes related to the sublethal concentration of chloride that is involved in transmembrane transport and ion homeostasis and can be key to the further development of transcriptomic tools for evaluating stress response. Overall, our study underscores the importance of understanding the molecular mechanisms underlying osmoregulation in the face of global freshwater salinization and provides a basis for further investigation into the impact of environmental stressors on fish populations.

Supporting information

S1 File. High-level gene ontology classification of the DEGs genes database. In this database, the differentially expressed genes of treatments A and C, compared to the control, are categorized into three main Gene Ontology categories and their corresponding high-level pathway categories.
(XLSX)

S2 File. Database of the significantly enriched gene ontology terms of the DEGs. Database of the complete list of enriched gene ontology terms for the different treatments, where the related genes are extracted and assigned to the correspondent gene.
(XLSX)

S3 File. Database of the DEGs related to osmoregulatory gene ontology terms. In this database all the genes that were identified to be related with an osmoregulation-related GO term are listed and detailed information is given.
(XLSX)

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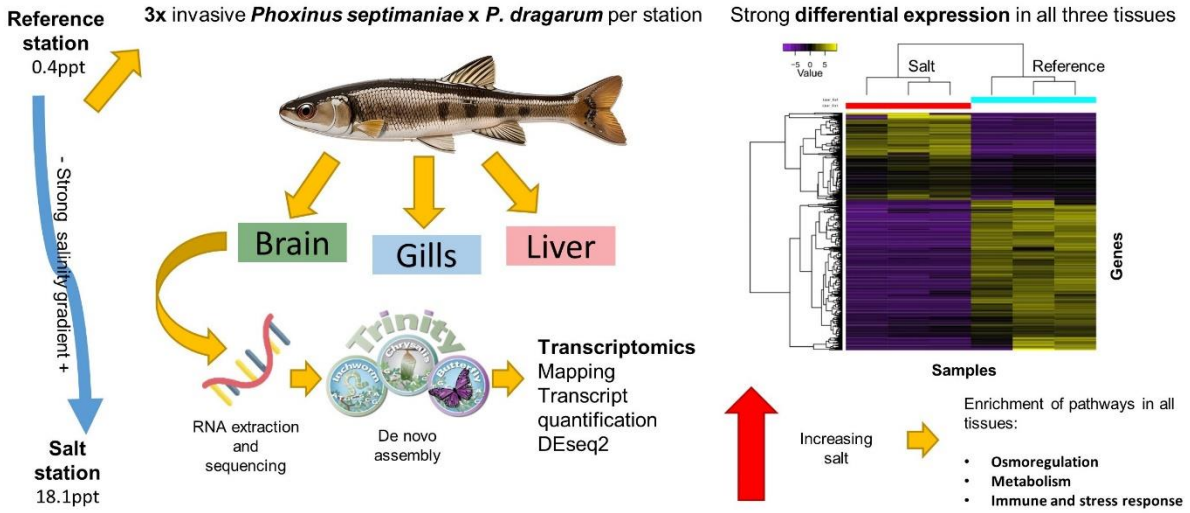
Chapter 2: Unravelling the molecular mechanisms of fish physiological response to freshwater salinization: a comparative multi-tissue transcriptomic study in a river polluted by potash mining

Graphical abstract:



Keywords: Freshwater salinization; Transcriptomics; Osmoregulation; Fish physiology; Minnow; Invasive

Highlights:

- Minnows adapt to salt stress, unveiling crucial molecular responses for survival.
- Transcriptomics reveal salinity stress in gills, liver, and brain pathways.
- The brain plays a pivotal role in stressor adaptation.
- Transcriptomic analysis suggests potential strategies in conservation.

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Unraveling the molecular mechanisms of fish physiological response to freshwater salinization: A comparative multi-tissue transcriptomic study in a river polluted by potash mining☆

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ABSTRACT

Freshwater salinization is an escalating global environmental issue that threatens freshwater biodiversity, including fish populations. This study aims to uncover the molecular basis of salinity physiological responses in a non-native minnow species (*Phoxinus septimaniae* x *P. dragarum*) exposed to saline effluents from potash mines in the Llobregat River, Barcelona, Spain. Employing high-throughput mRNA sequencing and differential gene expression analyses, brain, gills, and liver tissues collected from fish at two stations (upstream and downstream of saline effluent discharge) were examined. Salinization markedly influenced global gene expression profiles, with the brain exhibiting the most differentially expressed genes, emphasizing its unique sensitivity to salinity fluctuations. Pathway analyses revealed the expected enrichment of ion transport and osmoregulation pathways across all tissues. Furthermore, tissue-specific pathways associated with stress, reproduction, growth, immune responses, methylation, and neurological development were identified in the context of salinization. Rigorous validation of RNA-seq data through quantitative PCR (qPCR) underscored the robustness and consistency of our findings across platforms. This investigation unveils intricate molecular mechanisms steering salinity physiological response in non-native minnows confronting diverse environmental stressors. This comprehensive analysis sheds light on the underlying genetic and physiological mechanisms governing fish physiological response in salinity-stressed environments, offering essential knowledge for the conservation and management of freshwater ecosystems facing salinization.

1. Introduction

Human activities such as the use of salts for road deicing (Hintz & Relyea, 2019), resource extraction (Cañedo-Argüelles et al., 2013), and agriculture (Thorslund et al., 2021) are leading to an increase in the salinity of freshwater ecosystems around the world (i.e. freshwater salinization). Since freshwater organisms can only tolerate certain salinity ranges, freshwater salinization is leading to drastic reductions in aquatic biodiversity (Cañedo-Argüelles et al., 2019). However, our understanding of the ecological impacts of freshwater salinization is still limited. While fish have been identified as potentially affected by salinization, they have received considerably less attention than other freshwater organisms (Cunillera-Montcusí et al., 2022).

Potash mining operations often store their wastes in open areas near the mines, creating artificial salt mountains, primarily composed of NaCl, such as the case of our study site (an active potash mine situated near the Llobregat River in Barcelona, Spain). These mountains of salt-rich waste have the potential to generate highly saline effluents that are discharged into surrounding rivers and streams, becoming the main driver of freshwater salinization in various recorded locations (Otero & Soler, 2002). Although brines are usually collected and treated and/or transported to the sea, many technical problems persist (e.g. leaks in the collection systems, saline water infiltration) that can lead to severe salinization (Gorostiza et al., 2022; Gorostiza & Saurí, 2019). For example, in the Soldevila stream (NE Spain, very close to our study site) the electrical conductivity was 132.4 mS/cm (Ladrera et al., 2016),

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much higher than that of the seawater in the western Mediterranean [45 mS/cm]. These point source saline pollution effluents associated with potash mining activities are known to severely reduce the diversity of native freshwater macroinvertebrate species (Bäthe & Coring, 2011; Cañedo-Argüelles et al., 2017) and promote macroinvertebrate invasions (Arle & Wagner, 2013; Braukmann & Böhme, 2011; Lewin et al., 2018), but the effect on fish communities remains largely unknown.

Novel high-throughput methods such as transcriptomics offer promising advancements in evaluating the effects of stressors like salinity in wildlife studies conducted in field settings where multiple stressors act on stream fauna. The reliance on traditional methods, which measure single physiological parameters or a few genetic biomarkers, is limited to assessing the effects of a limited pool of pre-established targeted stressors. In urban-influenced rivers, several stressors such as nonpoint source pollution and climate variability act together, confounding the assessment of the physiological status of instream fauna (Kaushal et al., 2019; Schäfer et al., 2023). Transcriptomics can measure the physiological responses of species in the wild to various stressors, even those not known in advance of sampling, thus offering a comprehensive understanding of their physiological status and enabling the identification of main stressors (Connon et al., 2018; Jeffries et al., 2021; Lowe et al., 2017). Prior research has successfully utilized transcriptomics to study wild fish populations, identifying stressors that influence their physiological responses, with salinity often being found as a significant factor (Escobar-Sierra & Lampert, 2024; Jeffrey et al., 2023; Komoroske et al., 2016). By analyzing gene expression data obtained under natural conditions, researchers can elucidate the effect on known and novel molecular pathways and identify the prevailing stressors impacting organisms exposed to various stressors in the wild.

Although our study site, the Llobregat River, experiences various anthropogenic pressures, such as urban water runoff, wastewater treatment plant discharges, and agricultural effluents, freshwater salinization from potash mining stands out as one of the main stressors affecting aquatic fauna. Hyperosmotic stress poses significant physiological challenges to many freshwater animals, especially fish, impacting their osmoregulation processes (Guh et al., 2015). The gill, as a primary organ for osmotic homeostasis, has been the focus of osmotic stress transcriptomic research, revealing differential expression of genes associated with the immune system, membrane transport, and regulation pathways in diverse fish species exposed to varying salinities (X. Chen et al., 2021; Cui et al., 2019; Escobar-Sierra & Lampert, 2024; Guo et al., 2018a; Lee et al., 2020; Su et al., 2020a). Recent studies have also identified differential gene expression in the brain and liver of certain fish species under osmotic stress, emphasizing pathways related to immunity, reproduction, growth, osmoregulation, energy metabolism, oxidative stress, and ion transporters (Lin et al., 2020; Liu et al., 2018; K. Zhou et al., 2020). Even though transcriptomic resources for specific tissues in fish exist, comprehensive studies assessing multiple tissue-specific salinity responses have been seldom performed. This study aims to address this gap by conducting a comparative transcriptomic analysis focusing on gill, brain, and liver tissues under salinity stress.

The genus *Phoxinus*, presents a diverse array of species distributed across the northern hemisphere, particularly in Europe and parts of northern Asia (Palandačić et al., 2020). Known for their adaptability, these small freshwater fish populate various habitats from mountain streams to lowland rivers and lakes (Frost, 1943). Several *Phoxinus* spp. species have been introduced to new catchments, notably in the Llobregat basin an introduction event has created a hybrid species resulting from the translocation of *P. septimaniae* from southern France and *P. dragarum* from the Garonne River (Corral-Lou et al., 2019). The *Phoxinus* spp. invasions into several new catchments have led to ecological consequences, such as competition for food and predation on eggs, adversely affecting native species (Borgstrøm et al., 2010; Garcia-Raventós et al., 2020; Tiberti et al., 2019, 2022). Notably, their adaptability to high salinity in brackish waters, observed in certain coastal regions, may bolster their potential to invade upstream and colonize systems facing altered ecological conditions due to freshwater salinization. Similar adaptive advantages in increasing salinity scenarios have been described in other freshwater species, leading to expanded populations and distributions (Dobrzycka-Krahel & Fidalgo, 2023; Hudson et al., 2021; Olin et al., 2022; Piscart et al., 2011). Although *Phoxinus* spp. euryhalinity suggests they have the advantage of tolerating salinity changes, it has been estimated that the activation of the hyperosmotic osmoregulatory mechanisms for euryhaline fishes can account for 20–68% of their total energy expenditure (Bœuf & Payan, 2001; Komoroske et al., 2016). These costs may drive the need for physiological and behavioral optimization to minimize energy expenditure, resulting in narrow physiological tolerance windows and behavioral avoidance of salinities outside the optimal range (Dowd et al., 2010; Komoroske et al., 2016). However, there is still a lack of knowledge on the molecular mechanisms underlying this species response to salinity stress. Their invasive potential, salinization adaptability, and wide distribution make the minnow a valuable model to study the osmoregulation mechanisms of euryhaline species under changing salinity concentrations.

The primary objective of this study encompasses understanding the molecular underpinnings of salinity physiological response in *Phoxinus septimaniae* x *P. dragarum* (from now on minnow) populations inhabiting contrasting salinity conditions related to the potash mining activity in the Llobregat River in Barcelona, Spain. Our research seeks to unravel the specific effects of freshwater salinization on the gene expression profiles of brain, gills, and liver tissues, targeting the identification of tissue-specific physiological pathways amidst heightened salinity levels. The working hypothesis is that fish inhabiting salt-polluted environments should exhibit distinct and tissue-specific gene expression patterns related to salinity physiological response. To address this hypothesis, high-throughput mRNA sequencing and differential gene expression analyses were employed to explore the gene expression profiles of minnow populations exposed to salt pollution.

2. Material and methods

2.1. Sampling design

Two sampling stations with distinct salinity levels along the main channel of the Llobregat River in Barcelona, Spain were sampled on June 9, 2022. The salt-polluted station (from now on "Salt" in the figures) is located near the outflow of a potash mine operated by Iberpotash, SA. in the locality of Sallent, which continuously releases high salinity waste. In contrast, the "reference station" is approximately 5 km upstream from the Salt-polluted station, situated near the Balsareny locality (Fig. 1). At the time of sampling, the Control station had a water conductivity of 834 μS/cm, a salinity of 0.4 ppt, and a temperature of 17.5 °C. These are normal values for Mediterranean rivers and streams (Sánchez-Montoya et al., 2012), which have relatively high salt concentrations due to rock dissolution. In contrast, the Salt station (placed less than 10 Km downstream of the reference station) exhibited a conductivity of 2641000 μS/cm, a salinity of 18.1 ppt, and a temperature of 17.2 °C.

Minnows were sampled by electrofishing using a portable unit that generated up to 200 V and 3 A pulsed direct current (DC) in an upstream direction. The fish collection was approved by the Regional Government of Catalonia (Ref. AP/004). All procedures were conducted following the European Directive for animal experimentation (2010/63/EU). After capture, fish were promptly euthanized with an overdose of tricaine methanesulfonate (MS-222 at 1 g./L) followed by brain, liver, and gill tissue collection. The collected tissues were immediately fixed in RNAprotect (QIAGEN) and stored at −20 °C. In total, six fish samples were obtained, with three from the Salt station and three from the Reference station. The fish selected for sequencing were all males of the

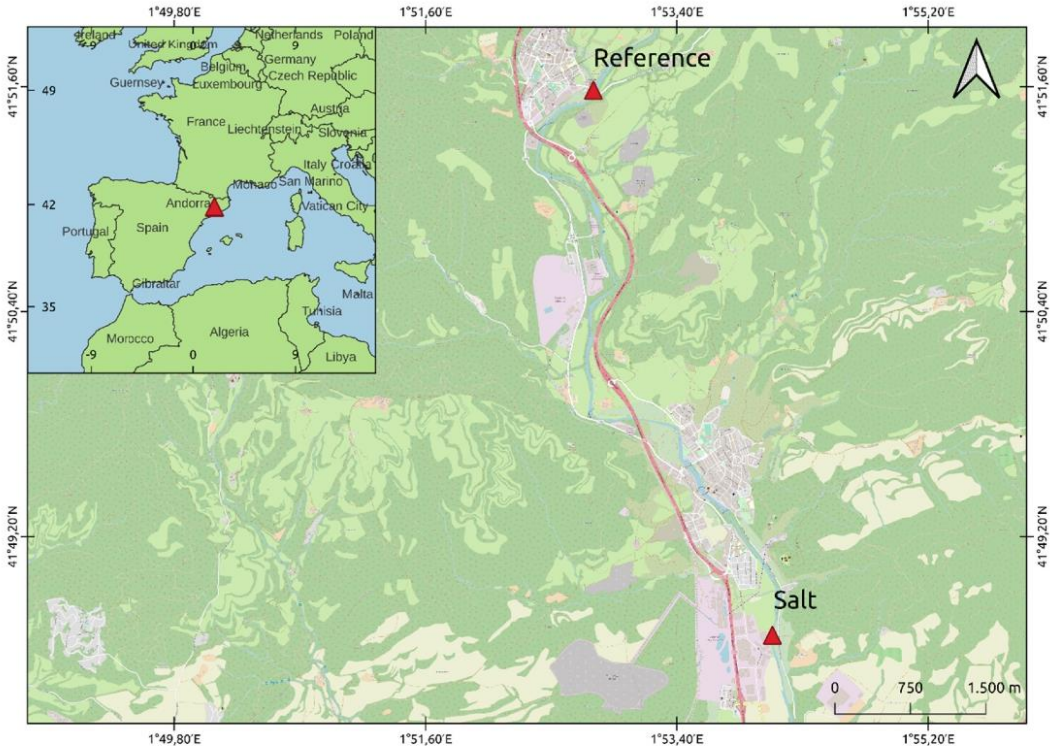

Fig. 1. Locations of fish sampling stations in the Llobregat River catchment. Three fish were collected from each station, and brain, gill, and liver tissues were collected for RNAseq. Salinity at the salt-polluted station "Salt" was much higher (2641000 μS/cm) than at the "Reference" station (834 μS/cm). Both stations were separated by a distance of 7.5 km.

same size class, with similar lengths (57.63 mm ± 14.47 mm).

2.2. RNA isolation and illumina sequencing

To isolate RNA, the fixed tissue of each sample was homogenized in a solution of 700 μl RLT buffer, with a 10 μl:1 ml concentration of β-mercaptoethanol, using a FastPrep-24™ (MP Biomedicals) bead beater for 30 s at 5 m/s. Subsequently, the RNA was extracted using a RNeasy Mini Kit (QIAGEN), following the manufacturer's instructions. The quality of the RNA extractions was confirmed using a Nanodrop 1000 Spectrophotometer (Peqlab Biotechnologie, Germany), ensuring a concentration >50 ng/μl, an OD 260/280 ratio of 1.8–2.1, and an OD 260/280 ratio >1.5. The RNA Integrity Number (RINe) value was assessed using the RNA ScreenTape system in an Agilent 2200 TapeStation, confirming that the samples had a RINe score greater than 7.0. Subsequently, an Illumina Tru-Seq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) was used to generate sequencing libraries, following the manufacturer's protocol. After purification, Illumina sequencing was performed on an Illumina/Hiseq-2500 platform (Illumina, San Diego, CA, USA) to generate 100 bp paired-end reads with a read depth of 50 million reads, as recommended by the Cologne Center for Genomics (CCG), Germany. The choice of read depth followed CCG technical advice, which estimated that this read depth would be sufficient for constructing a de novo transcriptome with eighteen biological replicates.

2.3. Sequence quality control, de novo assembly, and annotation

Quality control checks were conducted on the raw sequences of all 18 samples using FASTQC (Andrews, 2010). Subsequently, the quality was improved by filtering out adaptor sequences and low-quality reads using TRIMMOMATIC, with a quality threshold of phred +33, ensuring a minimum quality score of 25 for all bases, and retaining reads with a minimum length of 50 bp for downstream analyses (Bolger et al., 2014). TRINITY v2.9.1 (Grabherr et al., 2011) was used to create a de novo assembly with the nine pairs of clean sequences. The reads from the raw nine paired sequences were mapped to the de novo transcriptome using SALMON (Patro et al., 2017) as the abundance estimation method. To assess the completeness of the transcriptome assembly, BUSCO v5.2.2 (Simão et al., 2015) was used by searching against the vertebrata_odb10 dataset (Creation date: 2021-02-19). The expression matrix was generated, and low-expression transcripts (minimum expression of 1.0) were filtered using TRINITY v2.9.1, followed by the normalization of expression levels as transcripts per million transcripts (TPM). The identification of likely protein-coding regions in transcripts was performed using TRANSDECODER v5.5.0 (Haas et al., 2013). The filtered transcriptome sequencing reads were aligned to protein, signal peptides, and transmembrane domains using DIAMOND v2.0.8 (Buchfink et al., 2015), SIGNALP 6.0 (Teufel et al., 2022), TMHMM v2.0 (Krogh et al., 2001), and HMMER v3.3.2 (Finn et al., 2011). The de novo transcriptome was functionally annotated using TRINOTATE v3.2.2 (Grabherr et al., 2011).

2.4. Differential gene expression analysis

Prior to conducting differential gene expression analysis, the SALMON tool was used to align the clean reads of the three sites to the filtered transcriptome and calculate the mapping rate. The mapping tables were merged based on the stations and normalized using TMM (Robinson & Oshlack, 2010). The differential expression analysis, with three biological replicates for the three types of tissues, was performed to compare the Salt and Control stations using the DESeq2 package (Love et al., 2014). A fold change cutoff at >2 and an FDR≤0.05 was set. This rigorous analysis allowed us to identify thousands of gene expression differences between the tissues at the control and salt stations. The incorporation of the false discovery rate (FDR) within DESeq2 is essential to control type I errors, making it a widely accepted standard in genomics for correcting false positive results in differential expression analysis (Benjamini & Hochberg, 1995; J. J. Chen et al., 2010). The differentially expressed genes (DEGs) were visualized using a heatmap plot created with GGPLOT2 (Wickham, 2016), highlighting the changes in expression levels for each tissue type at both stations. All the significant DEGs were classified according to their higher-level Gene Ontology (GO) terms. Subsequently, a GO pathway enrichment analysis using ShinyGo (Ge et al., 2020) (version 0.8) was conducted to identify GO terms with unbalanced distributions between the two groups of genes or probe sets. To account for multiple testing issues, an FDR correction to the P-values was applied in this comparison to control falsely rejected hypotheses (Khatri et al., 2012). The top pathways identified in both the higher GO terms DEGs classification and the enrichment analysis were graphically represented, focusing on their relevance to osmoregulation challenges based on previous studies. The assembly was performed in the High-performance computing system of the University of Cologne (CHEOPS), and the rest of the analysis was carried out using the Galaxy project platform (The Galaxy Community et al., 2022).

2.5. RNA-seq qPCR validation

To validate the RNA-seq results, a subset of twenty-four genes from the DEGs dataset was selected (Table S1) and their expression assessed using real-time quantitative PCR analysis (qPCR). The sequences were retrieved from the assembled transcriptome and used to design specific oligonucleotides with the Primer-Blast software (Ye et al., 2012). First, reverse transcription of the total RNA samples was performed using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific), and the resulting cDNA was diluted tenfold for qPCR. The qPCR was performed using PowerUp™ SYBR™ Green Master Mix in a StepOne Real-Time PCR system following the manufacturer's indications. Each gene was assessed using the $2^{-\triangle\triangle Ct}$ method, with *ACTB* as the housekeeping gene for normalization. The qPCR results were expressed as $\log_2$ fold changes and conducted a Spearman's correlation analysis across all the samples to compare the expression values obtained from the RNAseq analysis.

3. Results

3.1. Sequence quality control, de novo assembly, and annotation

The RNA sequencing conducted on brain tissues yielded an average of 26.22 Mbp (±2.14 Mbp SD) total transcripts after quality control. For gill tissues, the total transcripts averaged 19.77 Mbp (±4.29 Mbp SD). Liver tissues had an average of 31.22 Mbp (±6.09 Mbp SD) total transcripts. Notably, all retained reads after quality control had a quality score %Q30 of 100%. The assembly with all sequences produced a raw transcriptome of 708.3 mb with an N50 of 1579 bp. The number of putative genes for the Trinity assembly was 434823, with a total transcript number of 760990. 3354 complete and fragmented BUSCOs were identified with an annotation completeness of 97.3%. Following the filtration of low-expression transcripts, 35.62% of the initial raw transcriptome was retained, resulting in a filtered transcriptome of 280.1 mb, encompassing 155426 transcripts with an N50 of 1930 bp. Notably, 3354 complete BUSCOs were retained, with an assembly completeness of 66.00%. Out of the de novo transcriptome, 90104 transcripts were annotated using TRINOTATE, representing 42.03% of the total transcriptome.

3.2. Differential gene expression analysis

The analysis revealed considerable alterations in global gene transcription profiles induced by changes in salinity across the brain, gills, and liver tissues of the minnow. The average percentage of reads mapping as proper pairs was 48.82% (±1.43% SD) for the brain, 48.95% (±1.16% SD) for gills, and 64.35% (±4.67% SD) for liver tissues. The principal component analysis (PCA) indicated tissue-specific expression patterns, showing that samples across both stations clustered together by tissue type (Fig. 2A). Along the PC1 (70.78% variance explained), the sequences were segregated based on tissue type, clearly delineating between brain, gills, and liver tissues. PC2 (9.61% variance explained) underscored stark differences in total transcription signatures between brain tissue and the other tissues. When examining the differential gene expression between the salt-polluted and the reference stations, significant numbers of genes were identified for each tissue. In gills, 286 DEGs (117 down- and 170 up-regulated) in the salt-polluted compared to the reference station were found (Fig. 2B). For brain tissue, 1350 DEGs (743 down- and 605 up-regulated) were observed in the salt-polluted compared to the reference (Fig. 2C). In the liver, 673 DEGs (426 down- and 248 up-regulated) in the salt-polluted compared to the reference were found (Fig. 2D). Notably, across all three tissue DEGs, the pattern of expression was consistent among the three sample replicates per station.

The significant DEGs for all three tissues were assigned to three major Gene Ontology (GO) categories: biological process, cellular component, and molecular function. The DEGs were classified in a total of 147, 197 and 182 higher-level GO terms relative to the reference station for the gills, brain and liver, respectively (Table S2). Interestingly, when the higher-level gene ontology terms were analyzed with a Venn diagram for all three tissues a significant number of terms shared with all tissues were found (Fig. 3). In the biological process category, 68 terms were shared among all three tissues (Fig. 3). In the cellular component category, 24 terms were common, and in the molecular function category, a total of 26 terms were shared across all tissues.

Key terms related to osmotic and chemical stress were graphically presented, showing the shared and distinct terms among the three tissues (Fig. 4). Noteworthy terms included hydrolase activity, small molecule binding, carbohydrate derivative binding, transporter activity, transmembrane transporter activity, and channel regulator activity in the molecular function category. In the cellular component category, terms such as organelle membrane, cell junction, extracellular region, and synapse were common across all tissues. In the biological process category, significant terms included responses to chemical stress, catabolic processes, developmental process regulation, signaling, immune system processes, growth, reproduction, and methylation, all shared across the three tissues.

The complete set of genes that were found to be differentially regulated for all tissues in the salt station compared to the reference were included in the enrichment analysis (Table S3). The top 10 significantly enriched pathways and those found to be related to salinity and overall chemical stress for the different tissues are presented graphically in Fig. 5. In gills for the molecular function category, the most significant pathway was the "chloride channel inhibitor activity", constituted by two genes *WNK2* and *CFTR* (Fig. 5A). These two genes appeared as constituents of several other ion transport and channel regulation-related pathways annotated in the enrichment analysis together with genes like *KCNC, ANXA2, ANO10, and CUL5* (Table S3). In the cellular component category, the "endomembrane system" pathway had the highest number of genes, followed by "chromosome, telomeric

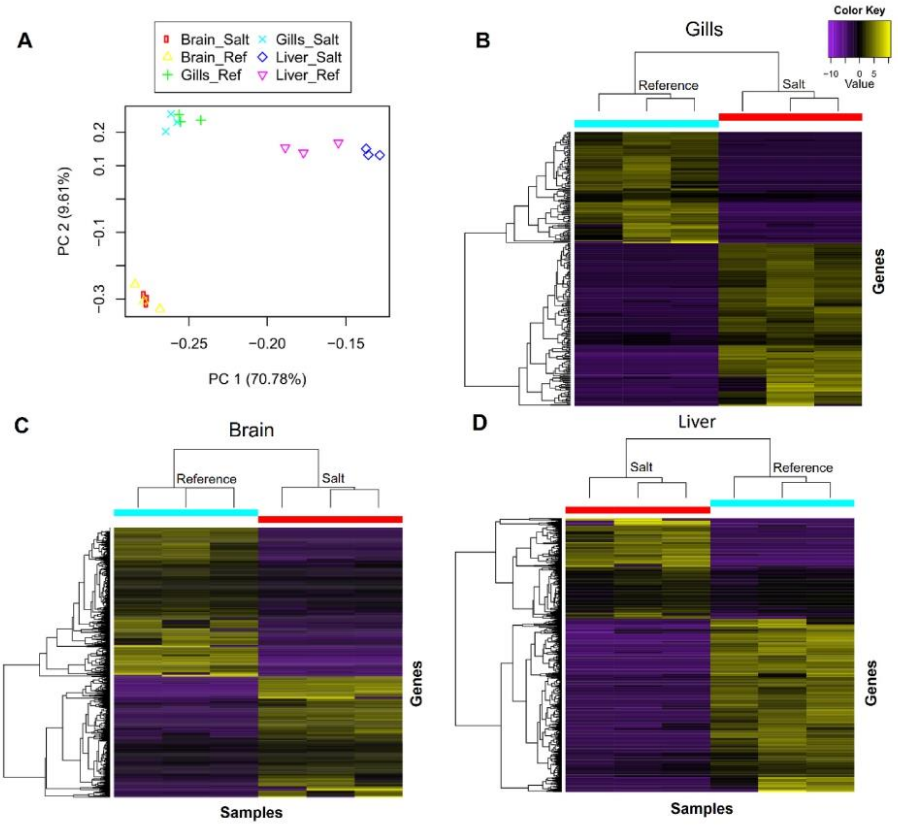

Fig. 2. A. Principal component analysis (PCA) of gills, brain, and liver transcriptomes of minnow (*Phoxinus septimaniae* x *P. dragarum*) sampled at a salt-polluted (Salt) and a reference station (Reference). **B.** Heatmap displaying 286 differentially expressed genes (DEGs) in gills between the salt and Reference stations. **C.** Heatmap displaying 1350 differentially expressed genes (DEGs) in the brain between the salt and Reference stations. **D.** Heatmap displaying 673 differentially expressed genes (DEGs) in the brain between the salt and Reference stations. For all heatmaps, the X-axis represents sample replicates per station, and the Y-axis represents individual gene expression. Upregulated genes are shown in yellow, with brighter colors indicating higher expression values. In contrast, purple shades indicate downregulated genes, with the brightest shade indicating the strongest downregulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

region", and pathways related to the vacuole and lysosome. In the biological process the pathways with the highest number of genes and higher significance were the "cellular response to stimulus", and "cellular response to stress", followed by pathways related to the transport of lipids.

In the brain, for the molecular function category, the pathway with the highest number of genes and more enrichment significance was "binding" followed by other pathways related to protein, ion, anion, and ATP binding (Fig. 5B). It is worth noting that several other pathways with less significance that were related to channel activity and overall transport were recurrent in the enrichment analysis with representative genes as *CLCN2, ANO1, KCNN3, KCNT1, KCNT2, RYR2, RYR3* (Table S3). For the cellular component, the "organelle" pathway presented the most genes, followed by "cell projection", other pathways related to neuronal activity like "neuron projection", "synapse and "axon" were significantly enriched. For the biological process the most significant enriched pathways were those related to development, followed by the "transport" pathway, and the response to organic substances and oxygen-containing compound. Interestingly the "growth", "neurogenesis" and "eye development" pathways were also enriched in the brain. A detailed examination of the differentially expressed genes in the brain revealed immune system-related genes such as *RPTOR* and *MKNK2*. It is worth noting that the "Fatty acid degradation" pathway was the only KEGG pathway that was found to be enriched in the analysis and it is represented in Fig. 6. In the figure, the red highlighted boxes represent the genes found in this study: *CPT1A*, *ACSL1* (6.1.2.3), *ACSL4* (6.1.2.3), *ACADM* (1.3.8.7) *ACADS* (1.3.8.1).

In the liver similar to our findings in the brain, for the molecular function category, the most important enriched pathways were related to binding, "anion binding" the one with more genes (Fig. 5C). In this category are also noteworthy the enrichment of the hydrolase and catalytic activity pathways. In the cellular component category, the more enriched pathways are the intracellular organelle and the cytoplasm. The enriched pathways "vacuole", "lysosome" and "lytic vacuole" are recurrent in the liver as it was in the gills. And the "B–WICH" complex enrichment is shared with the brain. For the biological process category,

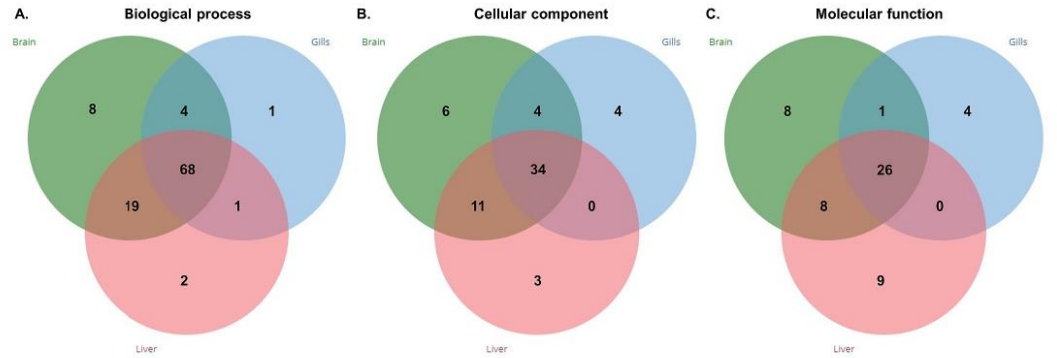

Fig. 3. Venn diagrams illustrating shared go higher-level terms across tissues. A. 68 terms shared in the Biological Process category. B. 34 terms shared in the Cellular Component category. C. 26 terms shared in the Molecular Function category. The overlap of gene ontology terms is represented by the intersection of circles, each corresponding to brain (green), liver (red), and gills (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

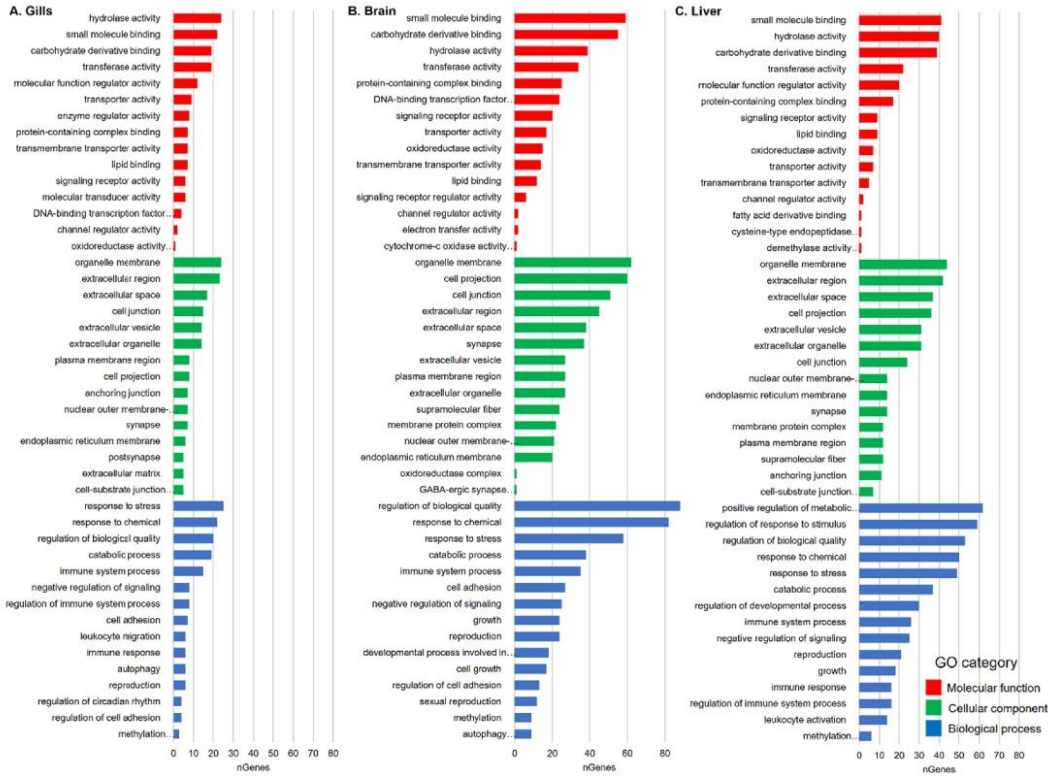

Fig. 4. Functional classification of differentially expressed genes in the high-level GO pathway categories across brain, liver, and gill tissues. The Y-axis represents the different GO general categories, with different colors for each one. And the X-axis represents the number of genes (nGenes) that were annotated to each pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

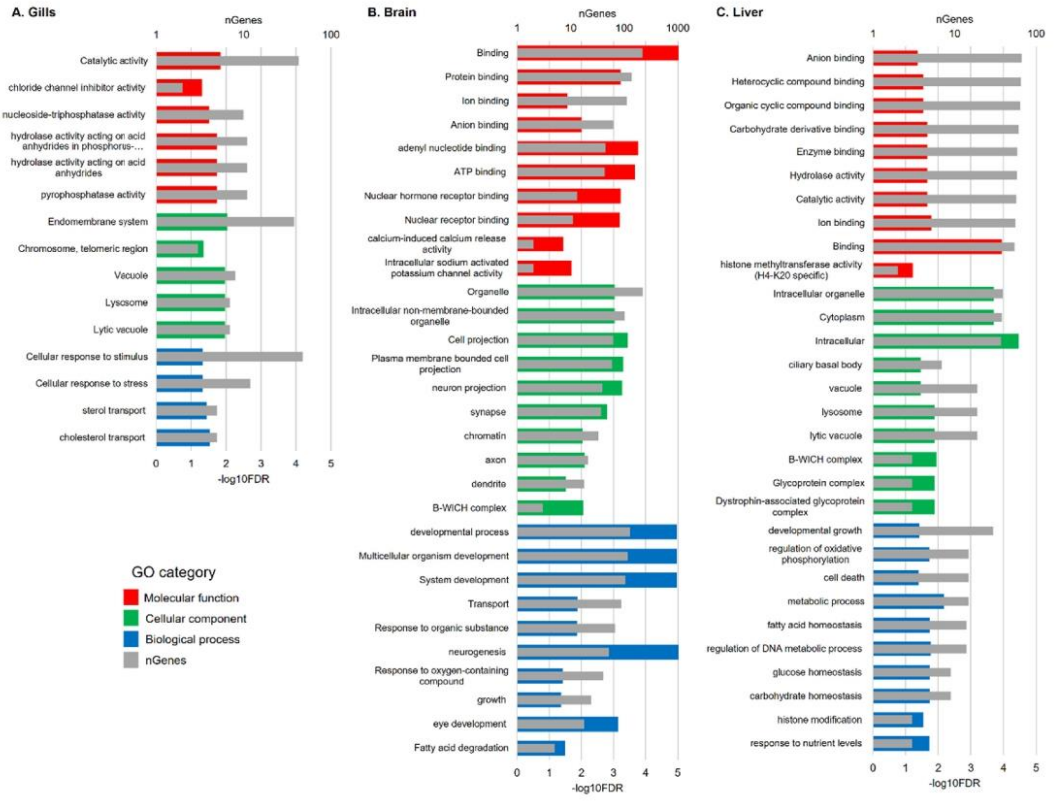

Fig. 5. Pathway enrichment analysis showing the top 10 enriched pathways related to salt or chemical stress across gills, brain, and liver tissues. The superior X-axis represents the number of genes annotated in the pathway (nGenes) and is expressed in log10 for the sake of the comparison between tissues. The inferior X axis is the enrichment FDR and is represented in –log10. The Y-axis is the top 10 enriched pathways related to salt or chemical stress. A. represents the gill-enriched pathways. B. the brain pathways and C. the liver.

the most enriched pathway was the "developmental growth", followed by the "regulation of oxidative phosphorylation" and "cell death". An enrichment of metabolic-related pathways was also evident with pathways such as "metabolic process", fatty acid, glucose, and carbohydrate homeostasis. Also, it is remarkable the enrichment of the "histone modification" and "response to nutrient levels". Additionally, the liver exhibited significant differential expression integral to immune-related pathways, notably including heat shock proteins (HSPs) such as HSP70, HSP7C, HSP7E, and the heat shock transcription factor 2 (HSF2).

3.3. RNA-seq data validation by qPCR

Validation of the transcriptomic data for the 24 chosen genes using qPCR was conducted using a Spearman correlation analysis of the log2 fold changes for the 18 samples analyzed on both platforms. Overall, there was a significant correlation between the log2 fold changes between platforms (Spearman, $R = 0.82$, $p < 0.001$; Fig. 7).

4. Discussion

This study reveals profound alterations in gene expression profiles induced by severe salinity stress conducting extensive RNA-seq analysis on the brain, gill, and liver tissues of the minnow. Notably, the differential gene expression patterns between the salt-polluted and reference stations demonstrated substantial differences across tissues, with the brain showing the highest number of differentially expressed genes. The GO and pathway enrichment analyses unveiled shared biological processes, molecular functions, and cellular components, emphasizing the impact of osmotic and chemical stress in all tissues. Specifically, the brain, gills, and liver exhibited tissue-specific responses to salinity changes, illuminating the intricate molecular physiological responses essential for osmoregulation and stress responses. Among the different tissues, the liver showed the greatest differential expression when the salt and control sites were compared. The validation of RNA-seq data through qPCR reinforced the robustness of the findings. Furthermore, the high quality of raw data and quality control results of our transcriptome, together with a mapping rate for all tissues on par with other studies (Guo et al., 2018b; Sun et al., 2020), demonstrate the effectiveness and reliability of the high-throughput sequencing transcriptomic analysis.

The overall differential expression of pathways, including responses to chemical stress, catabolic processes, developmental process regulation, signaling, immune system processes, growth, reproduction, and

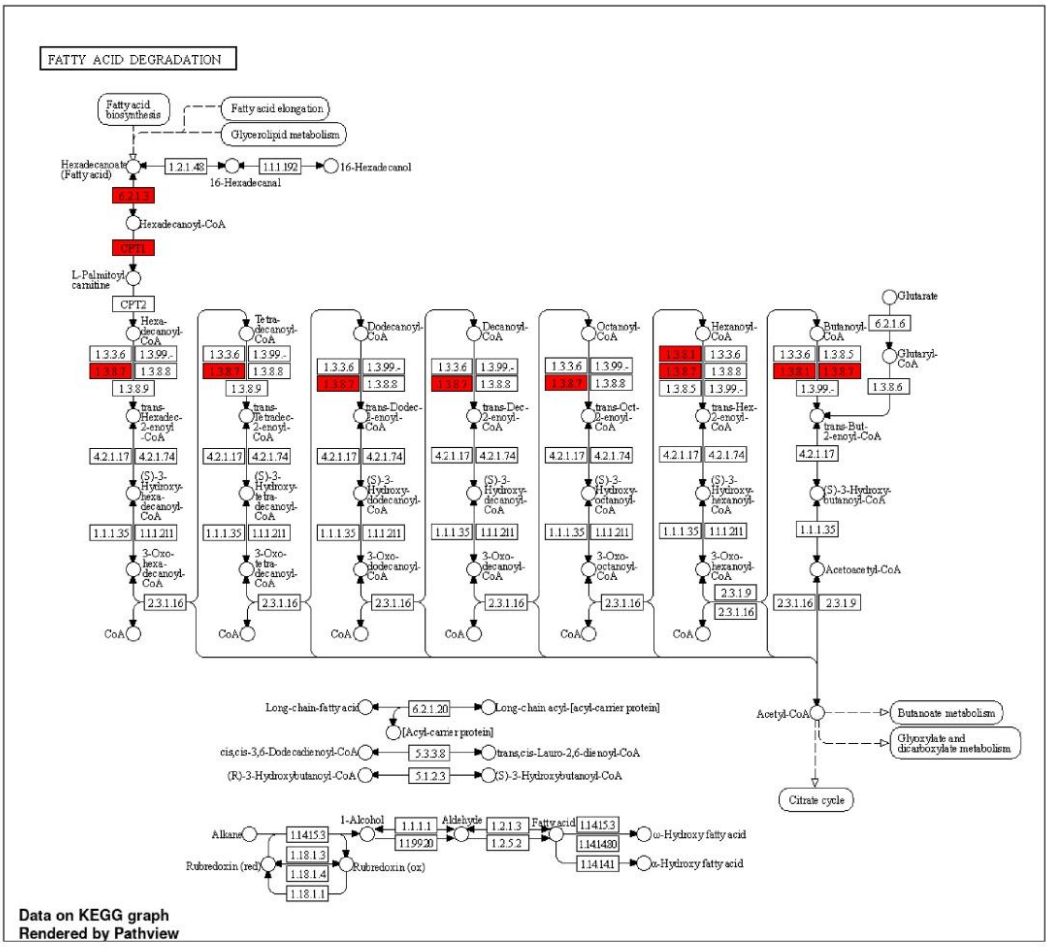

Fig. 6. KEGG enriched pathway "Fatty acid degradation" for brain tissue. Red highlighted boxes represent differentially expressed genes when comparing the salt and reference stations.

methylation, shared between tissues when comparing the contrasting salinity sites serves as an indicator of stress originating from potash mining effluents in the Llobregat River. From the various enriched pathways previously cited the ones related to differential expression of growth and reproduction which previous transcriptomic studies have linked to long-term salinity stress are highlighted (K. Zhou et al., 2020). Another set of relevant pathways is those related to neurogenesis and eye development which have been related to the effect of osmotic stress in previous transcriptomic studies (Gao et al., 2021; Politis et al., 2021; Zhu et al., 2023). Interestingly, the "methylation" pathway was enriched in all tissues, and in the liver, "histone modification". As suggested by a recent study coupling methylation and transcriptomics in euryhaline fish, this could indicate methylation changes and gene expression patterns under salinity stress (Blondeau-Bidet et al., 2023). As the current investigation is focused on the transcriptomic response of three tissues in a field setting where multiple stressors act simultaneously, and the complexity of the enriched metabolic pathways and their interplay increases, a thorough discussion of all enriched pathways would be beyond the scope of this study. The research discussion is therefore focused on osmoregulation, metabolic pathways, and immune responses, recognizing them as the primary pathways affected by our dominant salt pollution stressor.

4.1. Osmoregulation

Differentially expressed pathways related to osmotic stress and general chemical stress in all three gene ontology categories shared between all tissues under the salt-polluted station compared to the reference were identified. Notably, in the molecular function category, pathways such as transporter activity, transmembrane transporter activity, and channel regulator activity were of particular importance. These terms comprise proteins responsible for moving ions across membranes, ion channels, ion pumps, and ion transporters essential for maintaining osmotic pressure in exchange for considerable energy

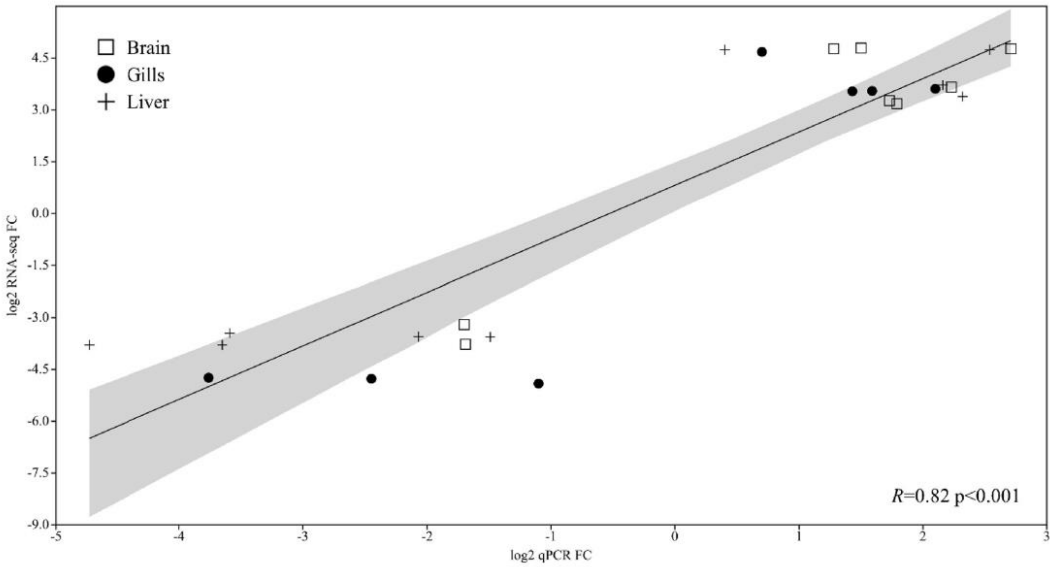

Fig. 7. Validation of RNA-seq data with quantitative PCR (qPCR) for the 24 genes analyzed on both platforms for the three studied tissues. Log2 fold changes for each gene are plotted, and the relationship was analyzed using a Spearman correlation. The reference line indicates a linear relationship between the RNA-seq and qPCR results. The shaded area represents a 95% confidence interval.

(Huang et al., 2004). Studies on the gills of various fish species in varying saline habitats have shown a significant number of DEGs involved in ion transport and transmembrane transport (X. Chen et al., 2021; Escobar-Sierra & Lampert, 2024; Gibbons et al., 2017; Guo et al., 2018b; Su et al., 2020a). Specifically, for gill tissue, an enrichment of osmoregulation-related pathways like "chloride channel inhibitor activity" was found. Genes constituting the main channel regulation-related pathways for the gills included *WNK2*, *CFTR*, *KCNC*, *ANXA2*, *ANO10*, and *CUL5*. Anoctamins, a family of Ca^{2+}-activated Cl^- channels and phospholipid scramblases, have been shown to support cell volume regulation (Hammer et al., 2015). *WNK2* regulates sodium-coupled chloride cotransporters and is part of an essential pathway for regulating cell volume in response to osmotic stress (Kahle et al., 2010). *ANXA2* is involved in cellular differentiation and ion channel conductance, with its differential expression recorded in euryhaline fish (Boulet et al., 2012). *KCNC*, like other voltage-gated Ca2+ channels, has been demonstrated to be involved in processes pertinent to the regulation of ion and water transport in animal epithelia—Ca^{2+} signaling and entry regulation (Zheng & Trudeau, 2015). The cystic fibrosis transmembrane conductance regulator (*CFTR*) is expressed on the apical surface of branchial ionocytes, serving as the conduit for active Cl^- secretion and an ion channel regulator in euryhaline fish (Shaughnessy & Breves, 2021). *CUL5* is a protein that mainly controls osmoregulation and regulates blood pressure within the body (Xu et al., 2021).

While the brain has not been traditionally studied concerning osmoregulatory stress, its high importance in osmotic homeostasis has been demonstrated, with the enrichment of osmoregulatory-related pathways reported under salinity stress in fish (Liu et al., 2018). In our study, the brain exhibited transport enrichment and overall osmoregulation, evident through the differential expression of genes like *CLCN2, ANO1, KCNN3, KCNT1, KCNT2, RYR2*, and *RYR3*. Chloride channels play a key role in the osmoregulatory physiology of all animals, with *CLCN2* differentially expressed in euryhaline fish exposed to osmotic stress (Bonzi et al., 2021; Su et al., 2020b). Another member of the anoctamin family, *ANO1*, related to osmoregulation, was differentially expressed in fish under osmotic stress (Taugbøl et al., 2022). *KCNN3* is a potassium-activated channel that can be differentially expressed in euryhaline fish exposed to osmotic stress (Bonzi et al., 2021). The potassium channels *KCNT1* and *KCNT2* are activated under high concentrations of chloride and can also be differentially expressed in euryhaline fish exposed to osmotic stress (Blondeau-Bidet et al., 2023; Lüscher et al., 2020). *RYR2* and *RYR3* are known interaction partners in the formation of chloride channels that are key in several physiological processes, including osmoregulation (Zeng et al., 2018). In summary, our results show that salinity from potash mining in the Llobregat is influencing the activation of pathways related to osmoregulatory stress and homeostasis in both gills and brain tissue. Furthermore, several candidate genes related to these pathways that could serve as biomarkers of this type of stress for further studies have been identified.

4.2. Metabolism

In all three tissues, differential gene expression related to metabolism when comparing the salt and control stations was observed, particularly notable in the liver, where various metabolic pathways were activated, and in the brain, with enrichment in the KEGG pathway 'Fatty acid degradation'. Osmoregulatory processes inherently demand energy, and the roles of lipid, glucose, and carbohydrate metabolism in fish osmoregulation have been extensively reviewed (Tseng & Hwang, 2008). Fish increase lipid metabolism primarily in the liver and brain during salinity stress, as reported in other transcriptomic studies of species facing hypersaline conditions (Gibbons et al., 2017; Hu et al., 2015). Specifically in the liver, enrichment of lipid metabolism under hypersaline stress for a euryhaline fish has included pathways such as fatty acid elongation, fatty acid metabolism, and fatty acid biosynthesis (K. Zhou et al., 2020). Both fish brain and liver tissues have shown an enrichment of pathways related to glucose and lipid metabolism when challenged with a strong

salinity gradient (Hu et al., 2015). Furthermore, some genes differentially expressed under osmotic stress in the brain, involved in the fatty acid degradation pathway, have been previously reported in studies of species undergoing osmoregulation stress. For instance, carnitine palmitoyltransferase 1 (*CPT1*) and overall lipid catabolism were found to be differentially expressed under osmoregulatory stress in the marine euryhaline crab *Scylla paramamosain* (Luo et al., 2023). *ACAD* genes, encoding acyl-CoA dehydrogenases, were differentially expressed under osmoregulatory stress in *Eriocheir sinensis*, an extremely invasive alien crab species (Hui et al., 2014). The enrichment of the fatty acid degradation pathway in the brain and other metabolic-related pathways in the liver in our study indicates that the minnow activates metabolic pathways to meet the energy demands associated with physiological responses required to survive hypersaline stress.

4.3. Immune and stress response

An overall differential expression of immune system processes and chemical stress across all tissues in the salt-polluted station was observed. The relationship between immunity and stress has been extensively reviewed, suggesting that long-term exposure to increased salinity significantly influences fish immune responses (Tort, 2011; Z. Zhou et al., 2021). In line with this, pathway enrichment and differentially expressed genes associated with stress and immune system regulation were identified. A notable finding was the significant differential regulation of a set of *HSP*s in the liver tissue when comparing the salt and control sites, including *HSP70*, *HSP7C*, *HSP7E*, and the heat shock transcription factor 2 (*HSF2*). These *HSP*s play a crucial role in translocation and protein folding, commonly serving as indicators and biomarkers of abiotic stress, expressed under salinity stress in several fish species (Jeffrey et al., 2023; Li et al., 2020; Lin et al., 2020; Puntila-Dodd et al., 2021). Another indicator of immune system alteration was the differential expression of the genes *RPTOR* and *MKNK2* in the brain tissue under salinity stress. The *MAPK* interacting serine/threonine kinase 2 (*MKNK2*) is a substrate of the *MAPK* pathway, crucial in its regulation (Maimon et al., 2014). The Mitogen-activated protein kinase (*MAPK*) signaling is known to be involved in various cell processes, including innate immunity (Roux & Blenis, 2004). Furthermore, its differential expression has been reported and linked to fish immune response when challenged with chemical stressors, including salinity (Duan et al., 2022; Tian et al., 2019). The regulatory-associated protein of *MTOR* complex 1 (*RPTOR*) is associated with the mammalian target of rapamycin (*mTOR*), which plays a key role in controlling and shaping the effector responses of innate immune cells (Weichhart et al., 2015). The activation of this pathway has been identified using transcriptomics in the euryhaline fish *Oreochromis mossambicus* following osmotic stress (Su et al., 2023). In conclusion, our findings highlight a comprehensive differential expression of immune system processes and stress-related pathways, emphasizing the intricate molecular responses of minnow to salinity stress in a polluted environment.

4.4. Ecological implications

Although our study design does not directly measure other stressors known to affect the Llobregat River, such as sewage discharges, agricultural runoff, and emergent pollutants altering fish fauna (Munné et al., 2012), our transcriptomic results enable us to infer physiological responses to severe salt pollution. Since potash mining effluents are mainly composed of NaCl (Cañedo-Argüelles et al., 2017), we suggest that the genomic responses detected were mainly triggered by salt stress.

Our study reveals the molecular physiology responses of minnows in the heavily polluted Llobregat, showcasing their resilience to salinity changes and anthropogenic stressors. This adaptability echoes traits seen in invasive species, suggesting their ability to thrive amidst new challenges (Davidson et al., 2011). The minnow's invasion success in the highly polluted Llobregat is documented by their three-fold increase in occurrence in the catchment between the 1990s and 2003, while the individual number and species diversity of native fish declined sharply at the same time (Maceda-Veiga et al., 2010). Our transcriptomic analysis sheds light on the molecular pathways activated by fish in response to the complex challenges of heavily polluted rivers. These challenges, stemming from human activities, serve as evolutionary pressures that select invasive species with traits like thermal and salinity tolerance (Cadotte et al., 2017). For instance, invasive fish species with increased thermal tolerance, salinity tolerance, and physiological adaptability—traits that enhance tolerance to urban environments—are prevalent in urban areas (Gomes-Silva et al., 2020; Green et al., 2023). This supports the proposition of Borden & Flory (2021), which suggests that urban areas create evolutionary pressures altering invasive species adaptations, increasing their potential to rapidly spread and succeed over native species. In the specific case of the Llobregat River, our results suggest that mitigating pollution from potash mining could help to control the invasion of *P. septimaniae* x *P. dragarum*.

5. Conclusion

In summary, this investigation explored the molecular mechanisms of freshwater salinization response in invasive minnow populations in the Llobregat River, Barcelona, Spain. Comprehensive changes in global gene expression profiles were observed across brain, gill, and liver tissues due to varying salinity levels between the salt-polluted and reference stations. The brain exhibited the highest sensitivity, with the most differentially expressed genes, underscoring its crucial role in salinity response. Tissue-specific pathways related to stress, reproduction, growth, immune responses, methylation, and neurological development were identified, all relevant under heightened salinity stress. Validation of RNA-seq data with quantitative PCR confirmed the reliability of these findings.

This study enhances understanding of the genetic and physiological mechanisms crucial for fish adaptation to salinity-stressed environments, extending previous fish transcriptomics research. It measured physiological responses to stressors in wild non-model organisms in field conditions, where multiple stressors may interact, emphasizing the study's importance. Highlighting the potential of transcriptomic approaches, the study demonstrates their value in evaluating responses to known or unknown stressors. This supports the assertion that transcriptomics is essential for guiding management and policy decisions, providing insights into complex stressor interactions, and enabling informed conservation strategies.

The analysis of tissue-specific pathways and shared biological processes elucidates the complexities of osmoregulation and stress responses in fish facing freshwater salinization. These findings present a detailed transcriptomic profile of adaptation, offering essential insights for the conservation and management of freshwater ecosystems under increasing salinity pressure. This study lays the groundwork for future research into the multifaceted impacts of salinity stress.

CRediT authorship contribution statement

Camilo Escobar-Sierra: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Miguel Cañedo-Argüelles:** Writing – review & editing, Resources, Investigation. **Dolors Vinyoles:** Writing – review & editing, Resources, Investigation. **Kathrin P. Lampert:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All relevant data are within the manuscript and its Supplementary data files, as well as at the GEO repository (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE271215.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.124400.

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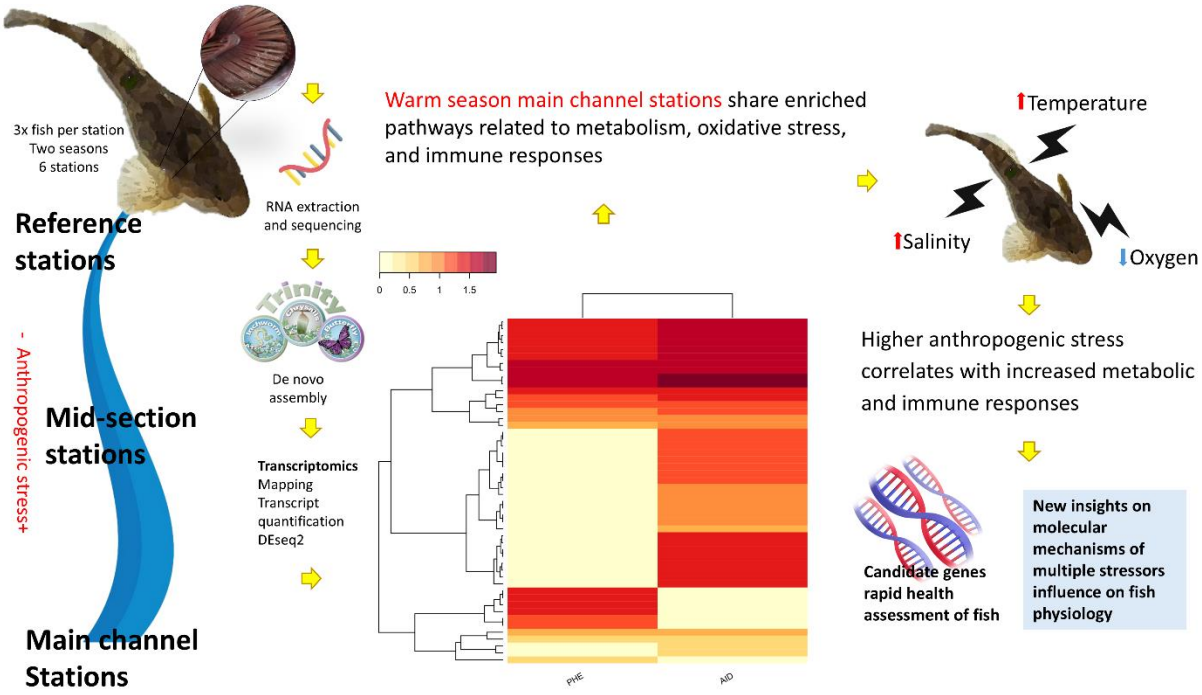
Chapter 3: Navigating the urban river: transcriptomic responses of freshwater fish to multiple anthropogenic stressors

Graphical abstract:



Keywords: Pollution; Gene expression; Aquatic Ecosystem; Physiology; Molecular ecology

Highlights:

- Higher anthropogenic stress correlates with increased metabolic and immune responses.
- Transcriptomics reveal seasonal variation in fish stress gene expression.
- Key stressors: high temperature, salinity, low oxygen impact physiology.
- Candidate genes identified for rapid health assessment of fish.
- Study highlights transcriptomics' role in conservation of endangered species.

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Navigating the Urban River: Transcriptomic Responses of Freshwater Fish to Multiple Anthropogenic Stressors

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Abstract:

Urbanization imposes multiple anthropogenic stressors on freshwater ecosystems, affecting aquatic species' physiological responses. This study explores the transcriptomic responses of the freshwater fish *Cottus rhenanus* to various stressors in an urban river system. RNA sequencing of fish from multiple stations revealed significant seasonal variations in gene expression, with a higher number of differentially expressed genes (DEGs) observed in the summer. Fish at the station experiencing the highest anthropogenic pressure showed notable responses, particularly during warmer months, with enriched pathways related to metabolism, oxidative stress, and immune responses. Key findings include the activation of metabolic stress pathways and immune system genes, such as IL-17 and MAPK pathways, influenced by high temperatures, salinity, and low oxygen levels. Pathway enrichment analyses highlight the impact of temperature and salinity on oxidative stress and osmoregulation, revealing the critical role of the transportome in adapting to salinity changes. These findings showcase the complex interactions between stressors and physiological responses, emphasizing the need for integrated conservation strategies to manage urban stream ecosystems.

Keywords: Pollution, Gene expression, Aquatic Ecosystem, Physiology, Molecular ecology

Introduction:

Urbanization is set to significantly increase, with the United Nations Population Division (UNPD) projecting an expansion of urban land cover by 1.2–1.8 million km² by 2030 (UNPD, 2019). This rapid growth has severe implications for freshwater habitats and biodiversity, a phenomenon often described as "Urban Stream Syndrome." This syndrome encapsulates various impacts on streams, including altered channel morphology, highly variable hydrographs, reduced biotic richness, and elevated levels of nutrients and contaminants (McDonald et al., 2019; C. J. Walsh et al., 2005). Additionally, urban streams suffer from nonpoint source pollution and climate variability, leading to complex "chemical cocktails" that act as multiple stressors (Kaushal et al., 2019; Schäfer et al., 2023). These multiple stressors pose significant threats to freshwater life, biodiversity, and ecosystem stability (Johnson & Penaluna, 2019), contributing to riverine biodiversity loss and altered community structures (Barrett et al., 2022). Environmental fluctuations in water quality, driven by anthropogenic stressors such as pH, salinity, temperature, dissolved oxygen (DO), and pollution, are recognized as major drivers of the distribution and health of fish species (Bernhardt et al., 2020; Menon et al., 2023).

The Ruhrgebiet region in Western Germany where the Emscher river flows, serves as a prime example of multiple stressors affecting urban streams. As one of Europe's most densely populated regions (Moos et al., 2021), it exhibits many factors associated with urban stream syndrome, such as channelization, reduced lateral and longitudinal connectivity, and poor water quality. Decades of wastewater discharge, coal mining, and heavy industrial activity have led to severe hydromorphological changes and pollution, with streams historically used as open sewers (Gerner et al., 2018). Coal mining has been one of the main stressors, significantly contributing to water quality issues, increasing chloride concentrations to levels as high as 3500

mg/L, far exceeding typical natural levels of less than 20 mg/L (Hintz & Relyea, 2019; Petruck & Stöffler, 2011). Although management measures and mine closures have reduced these concentrations to below 400 mg/L (Petruck & Stöffler, 2011), abandoned mines continue to contribute to freshwater salinization and chemical contamination (Schulz & Cañedo-Argüelles, 2019). To address this prolonged degradation, a large-scale restoration project initiated in 1992 aimed to improve water quality and reshape river morphology (Winking et al., 2016). As a result, some Emscher tributaries, such as the Boye, have been sewage-free since 2017, and significant improvements in channel morphology across the network have been achieved over the past 20 years (Gillmann et al., 2023). Chloride concentrations have also decreased in the Boye, although they can still be high reaching 26–286 mg/L, along with electrical conductivities between 0.4–1.93 mS cm^{-1} (Madge Pimentel et al., 2024). Despite these efforts, not all of the Emscher catchment has been restored, and main sections near Dortmund are still heavily influenced by urbanization, with high potential for increased runoff from road salts and sewage, further impacting water quality (Dugan et al., 2017).

Water quality is a critical factor for fish distribution and habitat suitability, influenced by variables such as temperature, conductivity, dissolved oxygen, and salinity. Conductivity, which correlates with the concentrations of ions like HCO3−, SO42−, and Cl−, tends to be elevated in disturbed catchments (Kefford et al., 2023). In urbanized environments, hypoxia (a significant global water pollution issue) exacerbates sublethal effects such as endocrine disruption and oxidative stress (Abdel-Tawwab et al., 2019; Pollock et al., 2007; Zhu et al., 2013). Fish, as ectotherms, are particularly vulnerable to temperature variability. Chronic temperature increases can compromise their ability to cope with additional stressors (Alfonso et al., 2021). Rapid temperature increases can induce acute stress responses, potentially impacting ecological dynamics. Freshwater salinization poses a significant threat by increasing

stress or mortality among freshwater organisms, thereby affecting biodiversity and ecosystem functionality (Cunillera-Montcusí et al., 2022). Freshwater fish, adapted to low ion concentrations, must expend considerable energy on osmoregulation, with increased salinity raising this energy expenditure (Guh et al., 2015; Tseng & Hwang, 2008). Elevated salinity impacts development, growth, and respiration, with fish often avoiding high salinity levels. Fish gills, in constant contact with the water, play crucial roles in oxygen uptake, osmotic regulation, temperature regulation, and immune response, making them sensitive indicators of physiological stress (Evans et al., 1999; Jeffries et al., 2021). The co-occurrence of multiple stressors in urban rivers makes it challenging to assess of their individual and combined effects (Orr et al., 2024). Furthermore, the complex interactions of multiple stressors complicate evaluating their effects on wild fish physiological responses under real-life conditions.

Traditional methods for evaluating the effects of multiple stressors, such as endpoint mortality, laboratory manipulations, and population studies, have limitations. High-throughput methods like transcriptomics offer a comprehensive approach to assessing gene expression in organisms exposed to multiple stressors (Lowe et al., 2017). Transcriptomics can provide detailed insights into the physiological status of species by measuring responses to multiple stressors (Jeffries et al., 2021). This approach has been successfully used in previous research to identify the stressors affecting wild fish populations (Escobar-Sierra et al., 2024; Escobar-Sierra & Lampert, 2024; Jeffrey et al., 2023; Komoroske et al., 2016). Gene expression data obtained under natural conditions can reveal the molecular pathways affected by stressors, helping to identify the specific stressors influencing the physiological response of fish in multiple stressor scenarios.

A species that is sensitive to multiple stressors is *Cottus rhenanus* (Markert et al., 2024a). In the Emscher catchment it was found in relicts, less affected river stretches but has been successfully reintroduced into several sites (Stemmer & Jacobs, 2015). *Cottus* generally require low water temperatures and high oxygen levels, restricting them to smaller, well-oxygenated cold streams (Brown, 1989; S. J. Walsh et al., 1997). Their sensitivity and conservation status in the Emscher catchment make them an ideal species to assess the effects of multiple stressors in urban streams.

The primary objective of this study, therefore, is to assess the physiological response of *Cottus rhenanus* to multiple anthropogenic stressors across the Emscher river catchment during both warm and cold seasons. This research aims to elucidate the relationship between these stressors and the gene expression profiles of gill tissue, identifying enriched physiological pathways and their interconnections. It is hypothesized that *Cottus rhenanus* in areas with higher anthropogenic stress will exhibit distinct gene expression profiles, enriched in pathways related to stress response, immune function, growth, and reproduction, regardless of the season. High-throughput mRNA sequencing and differential gene expression analyses will be used to examine these profiles along a gradient of stressors, with multivariate statistical methods exploring the relationships between gene expression and enriched pathways. This study employs de novo transcriptomic analysis to provide insights into the molecular mechanisms driving the physiological responses of a non-model wild fish species to multiple stressors in an urban stream environment.

Conducted under field conditions where multiple stressors interact, this research emphasize the importance of measuring physiological responses in wild, non-model organisms. By

integrating transcriptomic insights into broader ecological assessments and management practices, this study highlights the potential of transcriptomic approaches in evaluating responses to both known and unknown stressors. This approach demonstrates their value in assessing the overall status of wild fish and guiding conservation strategies. Ultimately, this research supports the assertion that transcriptomics is essential for providing insights into complex stressor interactions and enabling informed conservation strategies. It enhances our understanding of how freshwater species adapt to complex environmental challenges, and it paves the way for more accurate assessments of physiological status in organisms, with significant implications for conservation. Future research should focus on refining rapid screening tools using candidate genes and exploring the physiological responses to multiple stressors across various species and environments, thereby improving the management of endangered species under multiple anthropogenic stressors.

Material and methods:

Sampling design:

Six stations in wadeable sections of the Emscher river catchment were sampled for *Cottus rhenanus* in the warm (June) and cold periods (October) of the year 2022 (Figure 1) and a set of water chemistry, and hydro-geomorphological variables were taken. Water temperature, pH, conductivity, and dissolved oxygen (DO) were measured in the field using a multiparametric probe and shading, straightening and substrate diversity (SDV) were determined following the water framework directives and expert decision. Warter level at pole is the water depth in the mid-section of the transversal profile of stream. Water samples were taken in dark tinted bottles

and frozen under -20 °C and taken to the lab where the concentrations of nitrate, chloride and sulfate were measured. The table with values of the recorded variables is presented in Table 1.

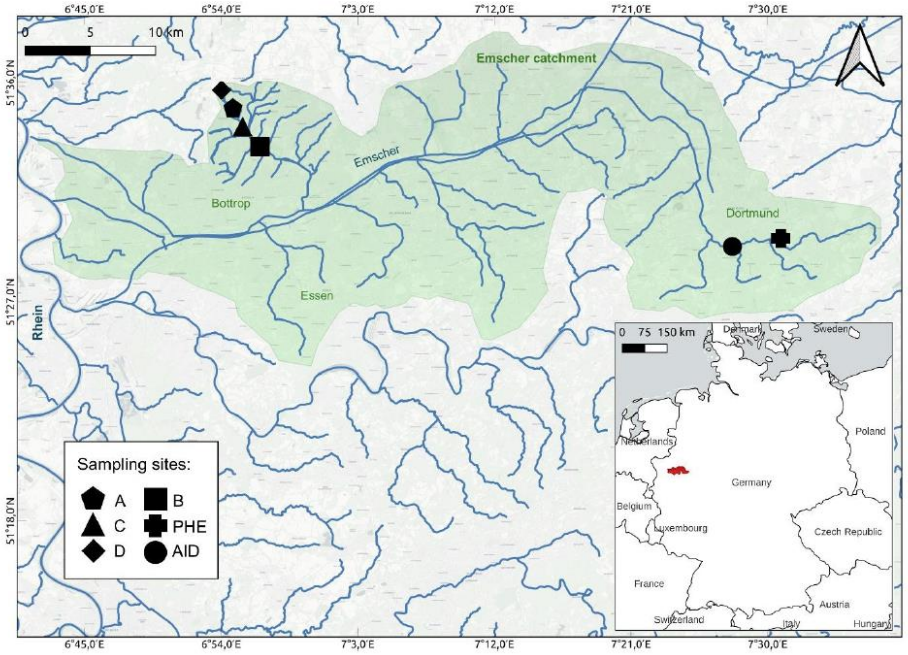

Figure 1. Fish sampling stations in the Emscher river catchment. Three fish were collected from each site for gill tissue RNAseq analysis. Sampling occurred once in the warm season (June) and once in the cold season (October).

Table 1. Summary of the stressor measured in all station in both seasons of sampling. Shading, straightening and Substrate diversity values range from 1 (best) to 5 (worst). Water level at pole measured in cm. Conductivity in µS/cm. Dissolved oxygen, nitrate, chloride, sulfate in mg/l.

	Station

	Stressor	AID	PHE	D	B	A	C
Warm	pH	7.6	7.7	8.27	8.34	8.05	8.43
	Conductivity	1002	820	551	828	599	627
	Dissolved oxygen	4.5	8.7	8.1	8.4	6.7	8.6
	Water temperature	16.7	19.2	13.8	15.6	14.1	14.2
	Water level at pole	85.0	44.0	21.5	23	20.3	15.5
	Nitrate	1.9	2.3	4.1	2.0	4.4	4.3
	Chloride	64	44	23	31	28	29
	Sulfate	110	130	72	141	83	85
	Shading	3	4	3	4	2	2
	Straightening	4	4	4	4	3	4
	Substrate diversity	3	2	3	4	2	3
Cold	pH	7.3	7.5	8.07	7.56	7.80	7.94
	Conductivity	1130	810	541	864	602	611
	Dissolved oxygen	5.5	8.8	9.0	9.4	9.4	8.4
	Water temperature	12.1	14	11.8	9.4	11.6	11.5
	Water level at pole	115.0	53	17.8	27.6	13.9	20.1
	Nitrate	4.19	2.2	4.9	2.1	3.9	4.2
	Chloride	104	43	23	29	28	27
	Sulfate	150	130	70	142	89	89
	Shading	3	4	3	4	2	2
	Straightening	4	4	4	4	3	4
	Substrate diversity	3	2	3	4	2	3

The stations were chosen to encompass the gradient of anthropogenic stress representative of the matrix of impacts that can be found in the Emscher catchment, from the mostly agricultural reaches in the headwaters and tributaries to some of the highly urbanized reaches in the main channel of river. Stations AID and PHE in the Emscher main channel and located in a densely urbanized area in Dortmund represent strong levels of anthropogenic stress. These stations exhibit the highest electrical conductivity, chloride, temperature, water levels, and sulfate, alongside lower dissolved oxygen, indicating significant stress in both cold and warm seasons. Station B is in the middle section of the Boye river, a tributary of the Emscher, this location has intermediate level of anthropogenic stress. Stations A, C and D are located towards the

headwaters of the Boye and represent some of the best water quality and lowest level of anthropogenic stress in the Emscher catchment (Figure 2 and Figure 3).

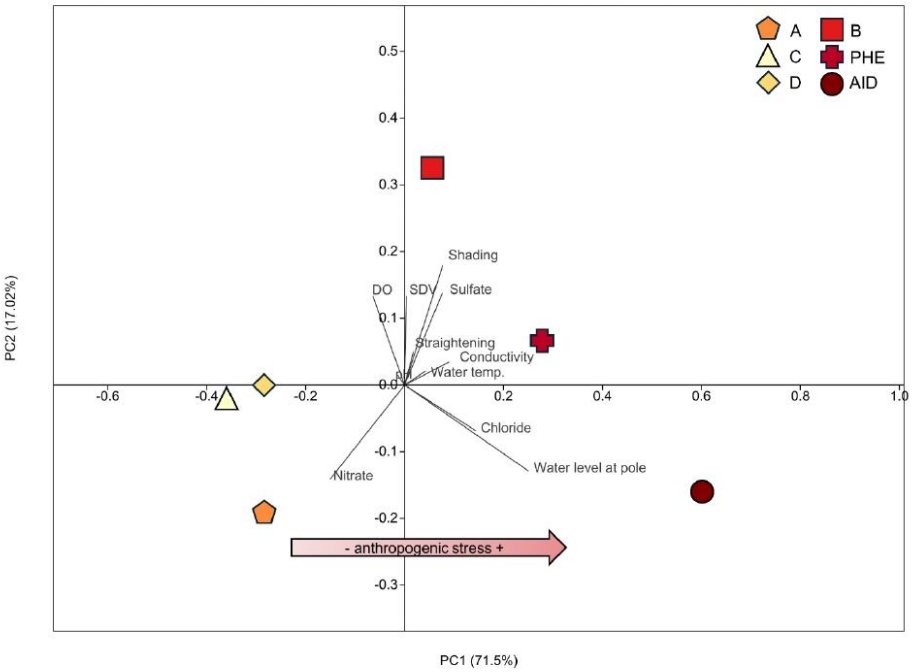

Figure 2. PCA of environmental variables in the Emscher river stations during the warm season. PC1 explains 71.5% of the variation and PC2 accounts for 17.02%. The bottom arrows indicate the gradient of anthropogenic stress across stations. Stations A, C, and D, characterized by higher dissolved oxygen and lower conductivity and chloride concentrations, are grouped to the left. Stations PHE and AID, with higher conductivity, chloride levels, water temperature, straightening, and lower dissolved oxygen, are grouped to the right. Station B represents mid levels of anthropogenic stress.

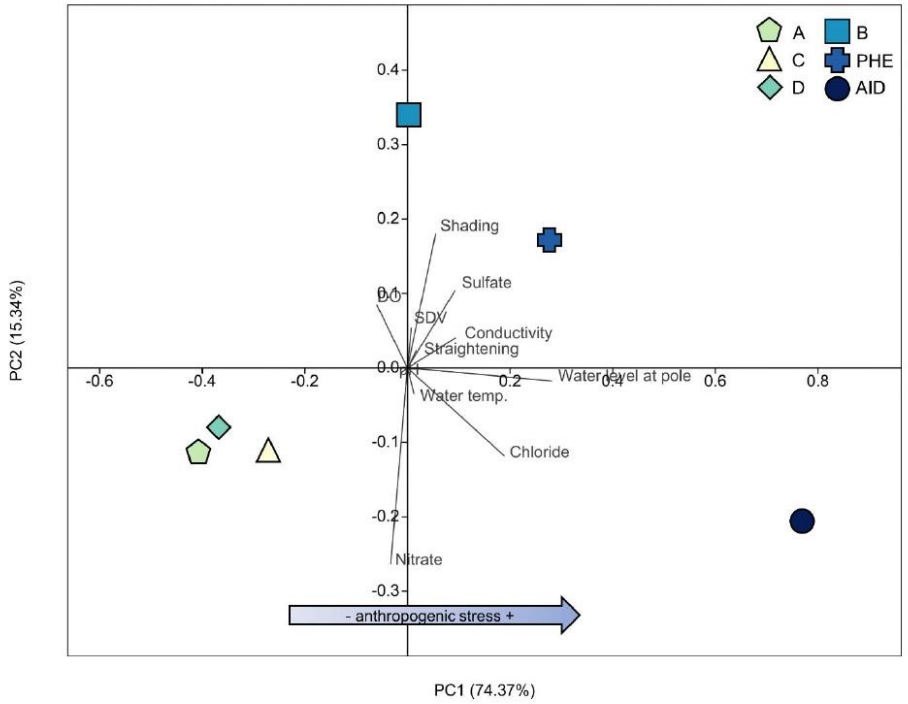

Figure 3. PCA of environmental variables in the Emscher River stations during the cold season. PC1 explains 74.37% of the variation and PC2 accounts for 15.34%. The bottom arrows indicate the gradient of anthropogenic stress across stations. Stations A, C, and D, characterized by higher dissolved oxygen and lower conductivity and chloride concentrations, are grouped to the left. Stations PHE and AID, with higher conductivity, chloride levels, water level, water temperature, straightening, and lower dissolved oxygen, are grouped to the right. Station B represents mid levels of anthropogenic stress.

Cottus rhenanus individuals were sampled using electrofishing equipment using a portable unit that generated up to 200 V and 3 A pulsed direct current (DC) in an upstream direction in all six stations in both the warm and cold seasons. All procedures were conducted following the European Directive for animal experimentation (2010/63/EU). Fishing and electrofishing

permit for stations A, B, C and D in the Boye river were given by the Bezirksregierung Münster ("DE 4407-301_Kirchheller Heide") and the sampling and permits in the Emscher main section stations (AID and PHE) were done directly by representatives of the local environmental agency (Emschergenossenschaft) and local fisheries office (Bezirksregierung Arnsberg- Obere Fischereibehörde). After capture, fish were promptly euthanized with an overdose of tricaine methanesulfonate (MS-222 at 1g./L) followed by harvesting the gill tissue, immediately fixing it in RNAprotect (QIAGEN), and storing it at -20°C. In total 36 samples were obtained, three from each station and season. The fish selected for sequencing were all males of the same size class, with similar lengths (67.77 mm ± 7.59 mm, N=36).

RNA Isolation and Illumina Sequencing:

RNA was isolated by homogenizing fixed gill tissue samples in 700 µl RLT buffer with a 10µl:1ml concentration of β-mercaptoethanol using a FastPrep-24™ bead beater for 30 seconds at 5 m/s. The RNA was extracted with the RNeasy Mini Kit (QIAGEN), following the manufacturer's instructions. Quality of RNA extractions was confirmed using a Nanodrop 1000 Spectrophotometer (Peqlab Biotechnologie, Germany), ensuring a concentration >50 ng/µl, an OD 260/280 ratio of 1.8-2.1, and an OD 260/230 ratio >1.5. RNA Integrity Number (RINe) values were assessed using the RNA ScreenTape system in an Agilent 2200 TapeStation, confirming RINe scores >7.0. Sequencing libraries were generated using the Illumina Tru-Seq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) per the manufacturer's protocol. Illumina sequencing was performed on an Illumina HiSeq-2500 platform, generating 100 bp paired-end reads with a read depth of 30 million reads, as recommended by the Cologne Center for Genomics (CCG), Germany, based on CCG's

estimation that this read depth suffices for constructing a de novo transcriptome with 36 biological replicates.

Sequence Quality Control, De Novo Assembly, and Annotation:

Quality control of the raw sequences from all 36 gill samples was performed using FASTQC (Andrews, 2010) to assess the initial quality. Subsequent quality improvement involved filtering out adaptor sequences and low-quality reads using TRIMMOMATIC, applying a phred +33 quality threshold, ensuring a minimum base quality score of 25, and retaining reads with a minimum length of 50 bp for downstream analyses (Bolger et al., 2014). A de novo assembly was constructed using TRINITY v2.9.1 (Grabherr et al., 2011) from the 36 pairs of clean sequences. Abundance estimation was carried out by mapping the reads of the raw nine paired sequences to the de novo transcriptome using SALMON (Patro et al., 2017). The completeness of the transcriptome assembly was evaluated with BUSCO v5.2.2 (Simão et al., 2015), utilizing the vertebrata_odb10 dataset (Creation date: 2021-02-19). An expression matrix was generated, filtering low-expression transcripts (minimum expression of 1.0) with TRINITY v2.9.1, and normalizing expression levels as transcripts per million transcripts (TPM). Likely protein-coding regions in transcripts were identified using TRANSDECODER v5.5.0 (Haas et al., 2013). The filtered transcriptome sequencing reads were aligned to protein databases, signal peptides, and transmembrane domains using DIAMOND v2.0.8 (Buchfink et al., 2015), SIGNALP 6.0 (Teufel et al., 2022), TMHMM v2.0 (Krogh et al., 2001), and HMMER v3.3.2 (Finn et al., 2011). Functional annotation of the de novo transcriptome was performed using TRINOTATE v3.2.2 (Grabherr et al., 2011).

Differential Gene Expression Analysis:

Prior to conducting differential gene expression analysis, clean reads from the six stations were aligned to the filtered transcriptome using SALMON to calculate the mapping rate. Mapping tables were merged according to stations and normalized using TMM (Robinson & Oshlack, 2010). Differential expression analysis was performed with three biological replicates for the gill tissues, comparing the reference station C to the rest of stations using the DESeq2 package (Love et al., 2014) with a fold change cutoff of >2 and FDR ≤0.05. This method identified significant gene expression differences between the gill tissues of all stations when compared the reference station, controlling for type I errors by incorporating FDR (Benjamini & Hochberg, 1995; J. J. Chen et al., 2010). Differentially expressed genes (DEGs) were visualized using a NMDS to explore the variation in the transcriptomic fingerprint of the different seasons, and the influence of the multiple stressors in their spatial variation. A GO pathway enrichment analysis was conducted over the differentially expressed genes DEGs using ShinyGo (Ge et al., 2020; version 0.8), applying FDR correction to account for multiple testing and control false positives (Khatri et al., 2012). The relevant pathways identified in the gene ontology and KEGG analysis were extracted to the database in supplementary material (S1). A canonical correspondence analysis CCA was performed to assess the relation of the anthropogenic stress variables and the magnitude of enrichment of the differentially expressed pathways. Assembly was executed on the high-performance computing system at the University of Cologne (CHEOPS), and the remaining analyses were performed using the Galaxy project platform (The Galaxy Community et al., 2022).

Results:

Sequence quality control, de novo assembly, and annotation:

The RNA sequencing conducted on gill tissue yielded an average of 33.48 (± 7.46 SD) millions of reads after quality control. The assembly with all sequences produced a raw transcriptome of 551.7 mb with an N50 of 2960 bp. The number of putative genes for the Trinity assembly was 265124, with a total transcript number of 416156. 3358 complete and fragmented BUSCOs were identified with an annotation completeness of 92.2%. Following the filtration of low-expression transcripts, 38.99% of the initial raw transcriptome was retained, resulting in a filtered transcriptome of 216 mb, encompassing 108328 putative genes with an N50 of 3092 bp. Notably, 2314 complete BUSCOs were retained, with an assembly completeness of 63.5%. Out of the filtered de novo transcriptome, 10000 transcripts were annotated using TRINOTATE, representing 61.62% of the total transcriptome.

Differential gene expression analysis:

When examining differential gene expression across the gradient stations and the reference station C, significant numbers of differentially expressed genes (DEGs) were identified for each station in both seasons. Overall, gene expression responses were stronger in summer, with a higher number of DEGs compared to the cold season. Station AID showed the strongest response in both seasons, with 1267 DEGs in the warm season and 770 DEGs in the cold season, likely due to higher stress levels at this station. In the warm season, station PHE had 622 DEGs, station A had 433 DEGs, station B had 585 DEGs, and station D had 971 DEGs. In the cold season, station PHE had 369 DEGs, station A had 653 DEGs, station B had 418 DEGs, and station D had 364 DEGs.

Non-metric Multidimensional Scaling (NMDS) analysis of the DEGs revealed significant changes in global gene transcription profiles induced by anthropogenic stress in *Cottus rhenanus* gills during both sampling campaigns. For both seasons, DEGs from biological replicates at different sites formed distinct groups in the NMDS plots (Figure 4 and Figure 5), confirming the quality and reliability of our sequencing. Notably, stations PHE and AID grouped together for both seasons, indicating a similar DEG signature. In the warm season (Figure 4), PHE and AID DEGs were influenced by shading, water level, dissolved oxygen concentration, and water temperature. In the cold season (Figure 5), PHE and AID DEGs were influenced by shading, water level, conductivity, and the concentrations of sulfate and chloride. This grouping pattern suggests that PHE and AID share a similar physiological response to high levels of anthropogenic stress.

In both seasons, station C's DEGs were on the opposite side of the NMDS plots from the PHE-AID group, reflecting the lower levels of anthropogenic stress at station C and validating its use as the reference station for differential gene expression analysis. Furthermore, stations A, B, and D were positioned at an intermediate distance between the PHE-AID group and station C. This organization can be explained by their intermediate levels of stress along the gradient between the reference conditions at station C and the highest anthropogenic stressors at stations PHE and AID. Notably, the stress values in both NMDS analyses were below the threshold of 2.0, allowing cautious interpretation of the relationships between DEGs and stressors. In summary, the differential gene expression patterns across the stations clearly reflect the varying levels of anthropogenic stress, with distinct grouping and stressor influences validated through NMDS analysis.

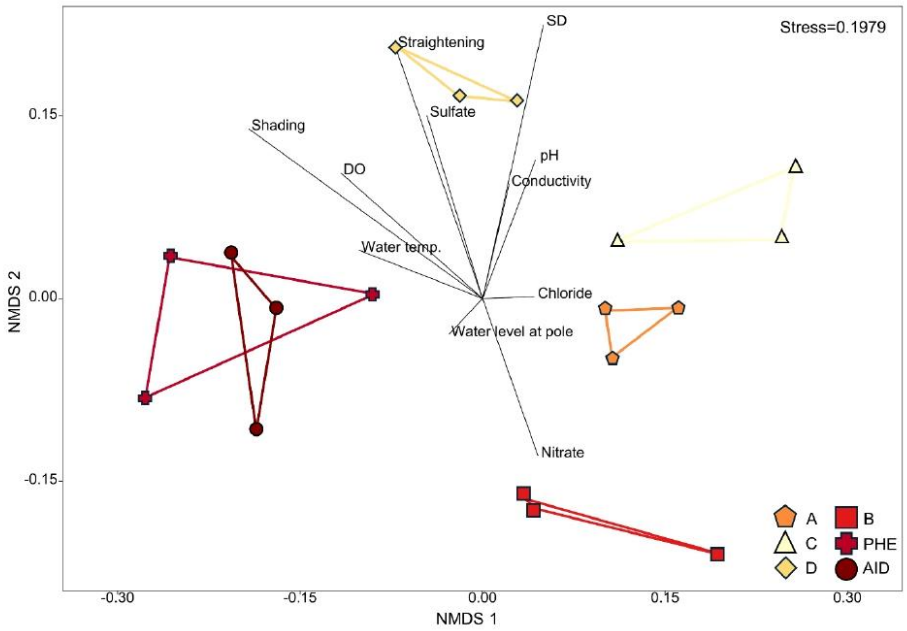

Figure 4. NMDS plot of DEGs and environmental variables in Emscher River stations (Warm season). Stress level is below the confidence threshold of 2.0 (0.1979). Biological replicates for each station form distinct groups. DEGs in Stations PHE and AID show similarity, influenced by shading, water level, dissolved oxygen, and water temperature. Station C samples group opposite the high stressor level stations PHE and AID.

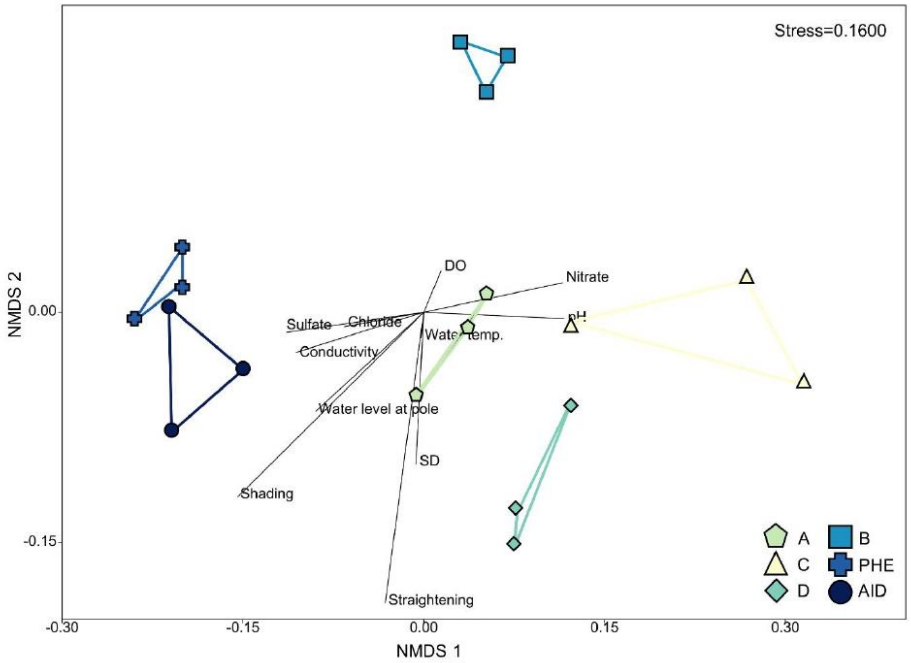

Figure 5. NMDS plot of DEGs and environmental variables in Emscher River stations (cold season). Stress level is below the confidence threshold of 2.0 (0.1600). Biological replicates for each station form distinct groups. DEGs in Stations PHE and AID show similarity, influenced by shading, water level, conductivity, sulfate, and chloride. Station C samples group opposite the high stressor level stations PHE and AID.

Overall, pathway enrichment was stronger during the warm period, with more DEGs enriched in both the KEGG and Gene Ontology databases across all samples. During the warm period, DEGs were significantly enriched (FDR $p<0.05$) for 2444 terms in the KEGG and Gene Ontology biological process, cellular component, and molecular component pathways. In the cold period, DEGs were significantly enriched (FDR $p<0.05$) for 1108 terms in the same pathways. Station AID had the most significantly enriched pathways in both seasons, with 809

in the warm period and 352 in the cold period. Conversely, station A had the fewest enriched pathways in the warm period (173), and station PHE had the fewest in the cold period (52) (Supplementary material S1).

The CCA explained 54.53% of the variation in enriched pathways across the different stations in relation to anthropogenic stressors during the warm season (CCA1: 28.73%, CCA2: 25.8%) (Figure 6). Enriched pathways for stations AID and PHE, the most stressed stations, were grouped together. This grouping was influenced by high water temperature, elevated chloride concentrations, high conductivity, and lower levels of pH, dissolved oxygen, and substrate diversity. Station D's enriched pathways were influenced by higher nitrate and dissolved oxygen levels, and lower sulfate and conductivity levels. Stations A and B's pathways were associated with higher pH, substrate diversity, sulfate, and conductivity. The CCA explained 61.32% of the variation in enriched pathways across the different stations in relation to anthropogenic stressors during the cold season (CCA1: 31.36%, CCA2: 29.96%) (Figure 7). Enriched pathways for stations AID, PHE, and B, the most stressed stations, were grouped together. This grouping was influenced by high water temperature, elevated chloride and sulfate concentrations, high conductivity, and lower levels of pH and nitrate. Stations D and A were characterized by higher levels of pH, substrate diversity, nitrate, and dissolved oxygen. In summary, CCA analysis revealed distinct patterns of pathway enrichment linked to specific

anthropogenic stressors, varying between the warm and cold seasons. Stations AID and B were consistently influenced by variables related to anthropogenic stress in both seasons.

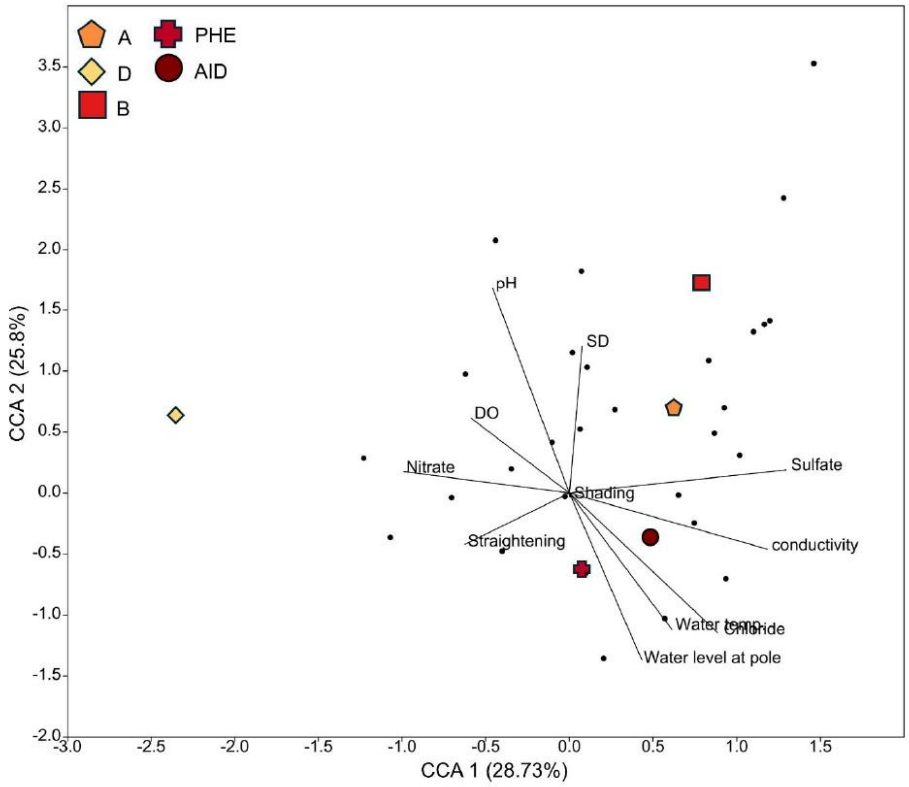

Figure 6. CCA plot of enriched pathways, and anthropogenic stressors for sampling stations during the warm season. The vectors represent the anthropogenic stressors. In colour and different geometrical shapes are the stations. The small black dots represent the gene ontology categories.

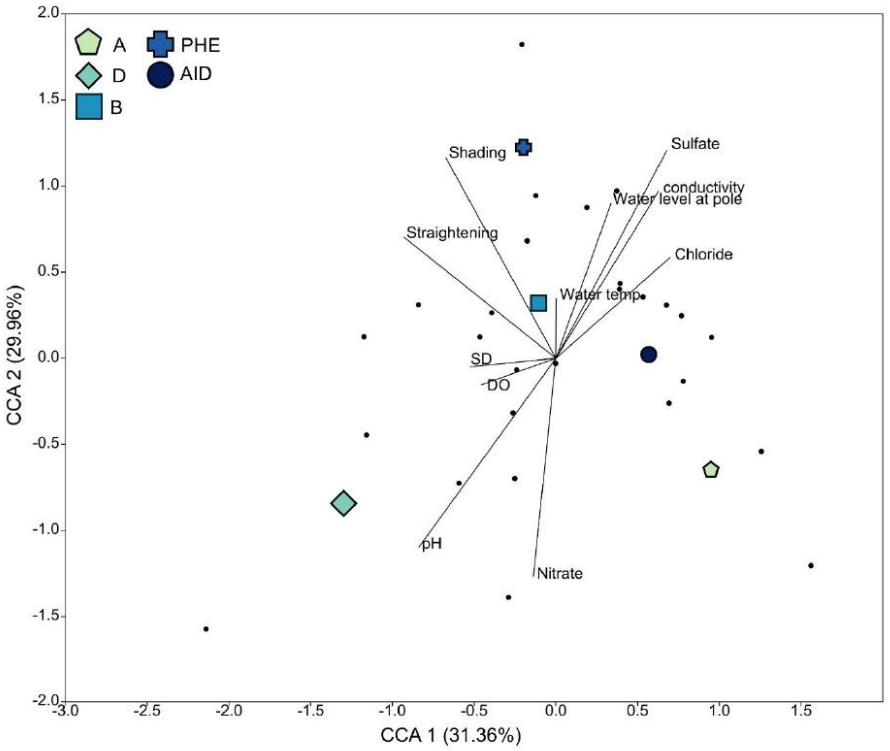

Figure 7. CCA analysis of KEGG, gene ontology categories, and anthropogenic stressors for sampling stations during the cold season. The vectors represent the anthropogenic stressors. In colour and different geometrical shapes are the stations. The small black dots represent the gene ontology categories.

Shared enriched pathways between stations AID and PHE encompass key parent GO groups such as Immune system process, regulation of transport, response to stress, and chemicals (Figure 8). Unique to station AID are pathways involving regulation of the MAPK cascade (GO:0043408, GO:0032872), regulation of ion transport (GO:0043270, GO:1901379) and circadian temperature homeostasis (GO:0060086), alongside distinct pathways related to

immune system processes and stress responses (GO:0050776, GO:0002252). Conversely, exclusive pathways identified in station PHE include those related to immune system processes (GO:0006952), growth (GO:0040007), and reproduction (GO:0000003). Noteworthy, the hyperosmotic salinity response pathway (GO:0042538) and renal system development (GO:0072001) were enriched, with many genes relevant to transport and osmoregulation. Genes associated with regulation of transport include *ANO6*, *ANO9*, *KCNH1*, *KCNC2*, *WNK2*, *WNK4*, and *ABCA1*, reflecting roles in osmoregulation, ion transport, membrane dynamics, signalling, and cellular organization. In chemical response pathways, genes participate in responses to inorganic substances, nitrogen compounds, and metal ions. Within the response to stress category, pathways such as inflammatory response, defence response, and MAPK cascade are enriched. Finally, the immune system process category prominently features pathways related to lymphocyte and leukocyte functions, indicative of significant immune response gene involvement. This heatmap analysis highlights the distinct and overlapping genetic responses of stations AID and PHE to anthropogenic stress during the warm season, emphasizing the overexpression of immune-related pathways, osmoregulation and transport pathways, and overall metabolism related to growth and reproduction.

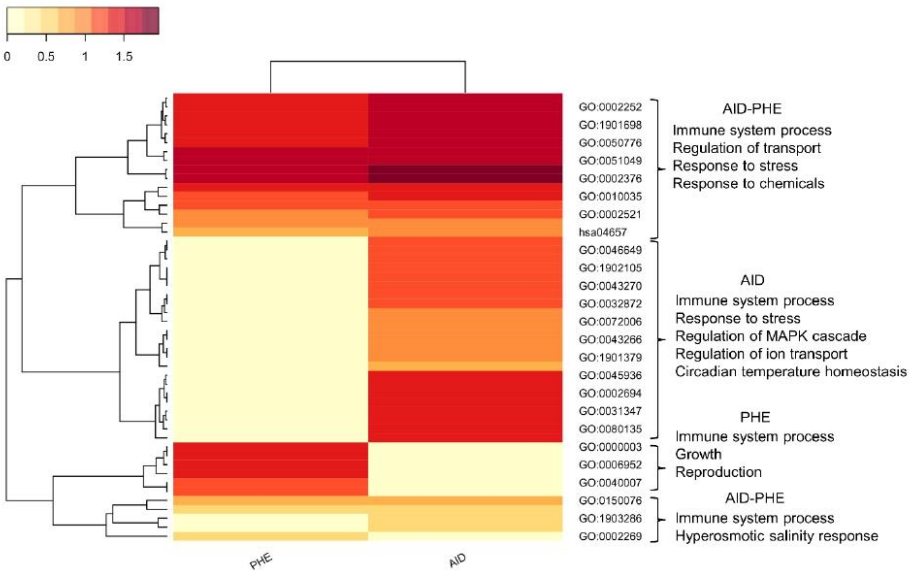

Figure 8. Heatmap of the number of genes for gene ontology and KEGG categories in high anthropogenic stress stations during the warm season. Stations AID and PHE are grouped based on shared gene ontology characterization and the number of genes per category. Brackets highlight the main categories found to be shared or unique to each station. The logfold number of genes has been transformed using Log10+1 for visualization.

Figure 9 presents a heatmap illustrating the distribution of genes across Gene Ontology (GO) and KEGG categories in high anthropogenic stress stations during the cold season, specifically stations AID, PHE, and B. The analysis reveals that the shared enriched pathways among these stations are primarily related to the parent GO category of developmental process. Station AID exhibits several unique pathways that reflect the influence of anthropogenic stressors, including response to stress, metabolic process, response to chemicals, and response to starvation. AID is also characterized by pathways related to the nervous system. Within these unique pathways, key genes associated with the response to oxidative stress pathway (GO:0006979) and overall

response to stress (GO:0033554, GO:0006950) include *NFAT5*. The response to starvation pathway (GO:0042594, GO:0009267) was enriched in the station AID. For the response to chemicals category, genes belong to pathways related to oxidative stress (GO:0034599, GO:0000302) and organic compound metabolic processes (GO:0006807, GO:0071704). Shared pathways between all three stations fall under the parent category developmental process (GO:0032502), with many pathways related to nervous system development (GO:0010721, GO:0048699, GO:0007399) and tissue development (GO:0007275, GO:0009888). The metabolic process category includes genes in pathways related to primary metabolic processes (GO:0008152, GO:0044238), with key marker genes such as *ACADS* and ACADL. Additionally, the pathway respiratory gaseous exchange by the respiratory system (GO:0007585) is notably enriched in station B. This heatmap analysis underscores the distinct and overlapping genetic responses of stations AID, PHE, and B to anthropogenic stress during the cold season, highlighting specific pathways and genes involved metabolism and stress response mechanisms.

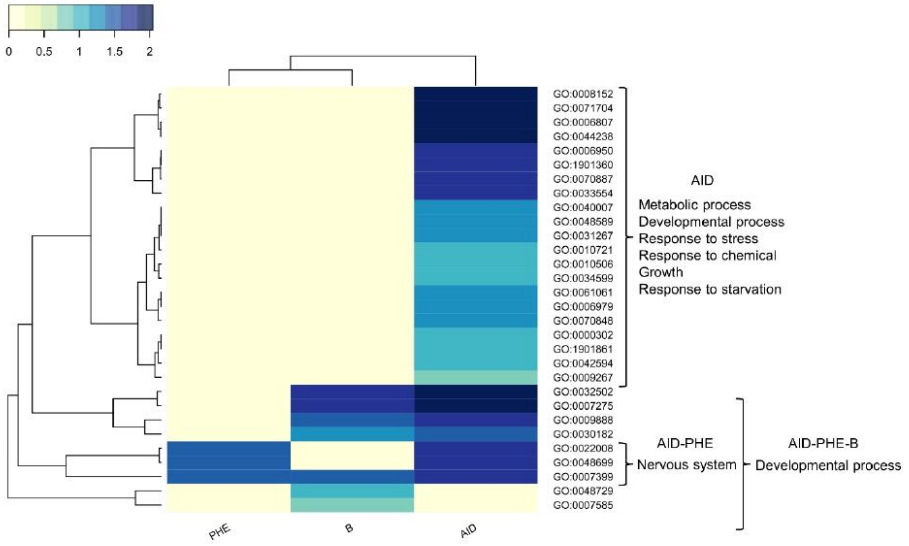

Figure 9. Heatmap of the number of genes for gene ontology and KEGG categories in high anthropogenic stress stations during the cold season. Stations AID, B and PHE are grouped based on shared gene ontology characterization and the number of genes per category. Brackets highlight the main categories found to be shared or unique to each station. The logfold number of genes has been transformed using Log10+1 for visualization.

The prevalence of immune response pathways in GO ontology for both PHE and AID prompted an in-depth analysis of enriched KEGG pathways using differentially expressed genes from both stations. This examination revealed pathways indicative of physiological stress, controlled by the expression of related genes. Notably, the MAPK (map04010) and IL-17 (hsa04657) signaling pathways were significantly influenced by environmental stress in the CCA analysis and included several DEGs. In the IL-17 signaling pathway, key DEGs identified were *IL1B* (Interleukin-1 beta, IL-1β), *TRAF6* (TNF Receptor Associated Factor 6), *NFKB1* (Nuclear Factor Kappa B Subunit 1), and *SOCS3* (Suppressor of Cytokine Signaling 3) (Figure 10). In

the MAPK pathway, the significant genes included *IL1B* (IL-1β), *NFKB1* (NF-kappa-B p105/p50), *TRAF6, JUNB* (Transcription Factor Jun-B), *EGR1* (Early Growth Response Protein 1), ATF4 (Activating Transcription Factor 4) and *SMAD4* (Mothers Against Decapentaplegic Homolog 4) (Figure 11). Notably, *IL1B*, *TRAF6*, and *NFKB1* were shared between both pathways, highlighting their importance as biomarkers in the response to environmental stress.

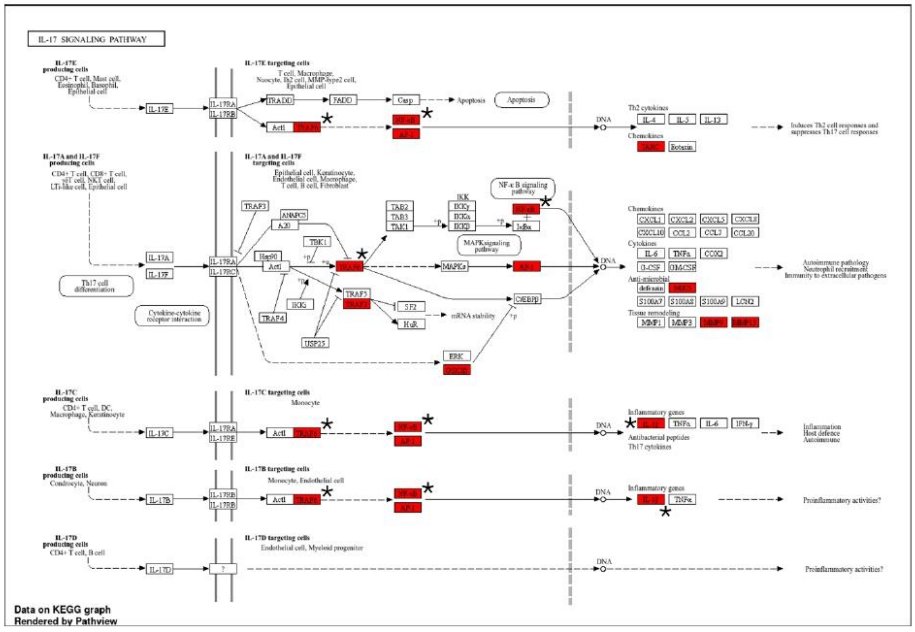

Figure 10. **KEGG enriched pathway "IL-17 signalling pathway" for the gill tissue of *Cottus rhenanus*.** Red highlighted boxes represent differentially expressed genes when comparing AID and PHE to reference station in summer. The genes marked with the symbol * are the ones shared between the IL-17 and MAPK signalling pathways.

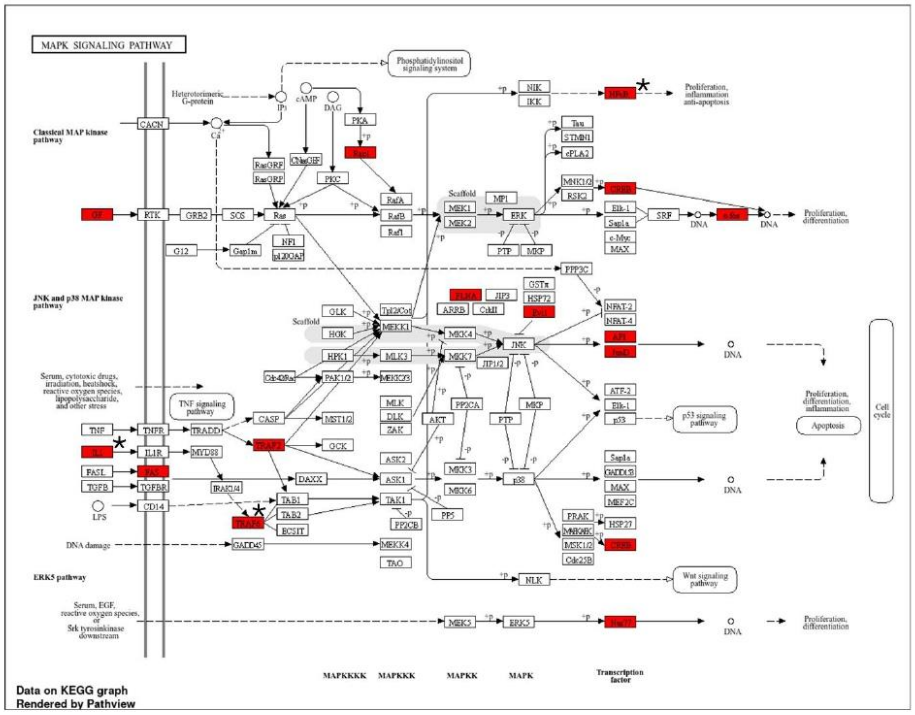

Figure 11. **KEGG enriched pathway "MAPK signalling pathway" for the gill tissue of *Cottus rhenanus*.** Red highlighted boxes represent differentially expressed genes when comparing AID and PHE to reference station in summer. The genes marked with the symbol * are the ones shared between the IL-17 and MAPK signalling pathways.

Discussion:

Our transcriptomic study offers a comprehensive analysis of *Cottus rhenanus* gills' physiological responses to anthropogenic stress, emphasizing the study's high data quality and key findings. High-quality RNA sequencing produced an average of 33.48 million reads per sample, resulting in a refined transcriptome assembly of 216 Mb with an N50 of 3092 bp, including 108,328 putative genes and 2314 complete BUSCOs, demonstrating 63.5% assembly

completeness. Differential gene expression analysis revealed significant seasonal variations, particularly at Station AID during the warm season with 1267 DEGs, and NMDS analysis confirmed distinct grouping patterns of DEGs among stations, indicating consistent sequencing quality and reliability. The transcriptomic approach facilitates the inference of physiological responses in wild fish to a gradient of multiple stressors in an urban river. The study reveals that these stressors collectively impact the differential expression of genes associated with metabolism, transport, oxidative stress, and chemical response pathways, leading to an enhanced immune response. This is evidenced by a significant number of DEGs related to immune function and the enrichment of genes in the IL-17 and MAPK pathways at stations with higher anthropogenic stress during the warm season. Given the complexity of the enriched metabolic pathways and their interactions, the discussion focuses on pathways related to the main identified anthropogenic stressors, metabolism, osmoregulation and transport, and the overall immune response. The discussion concludes by highlighting the broader implications of the findings for research on multiple stressors and animal conservation.

Metabolism:

In this study the gill tissue of *Cottus rhenanus* from the most stressed stations during both cold and warm seasons, revealed significant enrichment of pathways related to metabolic stress, reproduction, and growth. Several recent studies assessing the transcriptomic response of fish to different stressors have found functional responses in pathways broadly categorized under growth and reproduction, consistent with our findings (Beemelmanns et al., 2021; Du et al., 2014; K. Zhou et al., 2020). Notably, during the warm season, there was a marked increase in differentially expressed genes (DEGs) involved in cellular metabolic processes. In the cold season, the response to starvation pathway was prominently enriched in stressed stations. The

physiological responses observed align with previous studies highlighting strong metabolic reactions to similar stressors as it is discussed below.

One key stressor identified was salinity, particularly at stations with higher chloride concentrations and conductivities, where stress-related pathways were significantly enriched. Osmoregulatory processes are energy-intensive, involving lipid, glucose, and carbohydrate metabolism, as extensively reviewed by (Tseng & Hwang, 2008). Similar responses have been noted in other species; for instance, Milkfish (*Chanos chanos*) exhibit differential metabolic regulation in basal metabolism and oxidative phosphorylation under combined osmoregulatory and temperature stress (Hu et al., 2015). Additionally, hybrids of European minnow (Phoxinus sp.) show significant impacts on lipid metabolism under strong salinity stress (Escobar-Sierra et al., 2024). Temperature also influenced the stress transcriptomic response in both seasons, correlating with enriched pathways in lipid metabolism, as seen in juvenile turbot (*Scophthalmus maximus*) under temperature stress (T. Zhao et al., 2021). Lower dissolved oxygen levels in stressed stations further contributed to the enrichment of metabolic stress pathways, echoing findings in Yellow croaker (*Larimichthys polyactis*) (J. Wang et al., 2023).

Key biomarkers of metabolic stress were identified, including *ACADL* (Acyl-CoA Dehydrogenase Long Chain) and *ACADS* (Acyl-CoA Dehydrogenase Short Chain), which were differentially regulated in the AID station during the cold season. These genes, crucial in fatty acid metabolism, have shown differential expression under osmoregulatory stress in species such as *Eriocheir sinensis* and hybrid European minnows (Escobar-Sierra et al., 2024; Hui et al., 2014). The enrichment of the Cellular response to starvation pathway at the AID station during the cold season highlighted the stress endured. Notable genes within this pathway, such as *ATF4*, involved in glucose and lipid metabolism, were differentially expressed, consistent with findings in transgenic zebrafish and Japanese flounder under

temperature stress (Han et al., 2023; Yeh et al., 2017). Additionally, *WDR24* and *NPRL3*, regulators of the mTORC1 pathway, were highlighted due to their roles in cell growth and nutrient signal integration. The activation of this pathway has been linked to metabolism imbalance under various stressors in species like *Oreochromis mossambicus*, European minnows, Chinese perch, and chinook salmon (Escobar-Sierra et al., 2024; Su et al., 2023; Tomalty et al., 2015; P. Wu et al., 2020). During the summer, *SOCS1* and *SOCS3* genes, biomarkers of metabolic stress regulating insulin signaling and glucose metabolism, were notably differentially expressed in the AID station, aligning with findings in Arctic charr and Japanese flounder under dietary stress (Deng et al., 2018; Jørgensen et al., 2013).

These findings emphasize the significant impact of multiple stressors on the metabolic pathways of *Cottus rhenanus*, with osmoregulatory and temperature stress playing critical roles.

Hypoxia and oxidative stress:

In both cold and warm seasons, stations experiencing stress exhibited lower dissolved oxygen concentrations, influencing the enrichment of specific stress pathways associated with chemical responses and stress. Detailed analysis revealed significant enrichment of pathways and differentially expressed genes (DEGs) linked to oxidative stress, indicative of a hypoxic physiological response. Hypoxia induces chronic and acute stress responses in fish, leading to molecular, behavioral, morphological, physiological, and immunological changes (Abdel-Tawwab et al., 2019). The fish gill, crucial for gas exchange, undergoes significant remodeling and leads the organismal response to varying dissolved oxygen levels (Mitrovic et al., 2009; C.-B. Wu et al., 2017). Under hypoxic conditions, fish experience reduced oxygen consumption and accelerated oxygen transport (Honda et al., 2019). While bioindicators in fish remain within normal ranges under normal dissolved oxygen concentrations, acute hypoxic stress increases reactive oxygen species (ROS) production (Sun et al., 2020). Fish have evolved

an antioxidant defense system comprising enzymes and non-enzymatic molecules to regulate ROS homeostasis and adapt to hypoxia (Luo et al., 2021; Zhu et al., 2013). Failure of this system under hypoxic stress can exacerbate oxidative stress, tissue damage, apoptosis, or necrosis, contributing to the significant enrichment of oxidative stress-related pathways and DEGs observed in the gill tissue of *Cottus rhenanus* during both cold and warm seasons.

Transcriptomic studies investigating fish responses to hypoxia consistently identify the activation of oxidative stress-related genes and immune pathways. For example, in the silver carp gill, numerous DEGs indicate robust immune response and oxygen transport signaling under hypoxia (X. Li et al., 2022). Similar findings are reported in the yellow croaker liver and pearl gentian grouper, where pathways related to oxidative stress, apoptosis, and immunity are enriched (Liang et al., 2022; J. Wang et al., 2023). Notably, significant enrichment of the "Respiratory gaseous exchange by respiratory system" pathway at station B hints to the impact of dissolved oxygen stress. These results align with our findings, where alongside oxidative stress markers, we observed enrichment of immune responses indicated by IL-17 and MAPK pathway activation. The gill's role as an immune-competent organ with extensive mucosal surfaces, known as gill-associated lymphoid tissue, further supports these observations (Koppang et al., 2015). Additionally, oxidative stress's detrimental effect on fish immune and physiological functions is well-documented (Biller & Takahashi, 2018; Makrinos & Bowden, 2016; J. Wang et al., 2023).

Key candidate genes involved in oxidative stress and hypoxia were identified in the gill tissue of stressed stations across both seasons. Here we highlight some of these genes along with references to studies that have found them differentially expressed in response to hypoxia.

Examples include *G6PD* (Glucose-6-Phosphate Dehydrogenase), responsible for NADPH production crucial for ROS detoxification, and CCS (Copper Chaperone for Superoxide Dismutase), which activates *SOD1* to neutralize superoxide radicals (Lai et al., 2022). BMAL1, a circadian clock gene, and *CCN1* (Cyr61), involved in angiogenesis and cellular responses to hypoxia, were also differentially expressed (R.-X. Wu et al., 2023; H. Zhang et al., 2023). Additionally, *CDK1* and *CDK4*, regulators of cell cycle progression, showed enrichment in stressed stations during both seasons (Druker et al., 2021; He et al., 2017). *TRAF2* and *TRAF6*, mediators in TNF receptor signaling linked to oxidative stress and inflammation, exhibited high expression in stations stressed during the warm season (Jiang et al., 2023; G. Zhang et al., 2016).

In summary, the observed low dissolved oxygen levels in the stressed stations are leading to critical oxidative stress and hypoxia response pathways and biomarkers in *Cottus rhenanus* in the Boye River.

Osmoregulation and transport:

Freshwater salinity, driven by historical saltwater intrusion and urbanization, is a major stressor for *Cottus rhenanus* in the Emscher catchment. This study, along with previous research on three-spine stickleback in the same region, highlights that even low salinity levels can act as significant stressors. Even during the cold season, when osmoregulation and transport pathway enrichment was not paramount, key biomarkers of osmoregulation stress were recorded. These biomarkers, consistently found in various environments and different wild freshwater fish species under salinization stress, include *ANO6*, *ABCD1*, *ABCB6*, *SLC46A2*, and *NFAT5*. Anoctamins, such as *ANO6*, are involved in calcium-activated chloride channels and

phospholipid scramblases, supporting cell volume regulation. These have been shown to be differentially expressed under salinity stress (Escobar-Sierra & Lampert, 2024; Hammer et al., 2015; Taugbøl et al., 2022). *ABCD1* and *ABCB6* are part of the ABC gene family, the largest group of transmembrane transporter proteins in the human genome. They use ATP to facilitate the transport of various molecules across cellular membranes, maintaining cell homeostasis (Bieczynski et al., 2021). These proteins have been reported in previous transcriptomic studies of fish under salinization stress (Escobar-Sierra & Lampert, 2024). *SLC46A2*, like other solute carriers in teleost fish, is involved in transmembrane transport, generating counter-ion fluxes to maintain ion homeostasis while avoiding excessive membrane tension (Saric & Freeman, 2021; Verri et al., 2012). This gene was also expressed during salinity stress in the Emscher fort he three spine stickelback (Escobar-Sierra & Lampert, 2024). *NFAT5* is crucial in the cellular response to hypertonic stress, regulating genes that help cells adapt to osmotic changes, a response previously reported under osmotic stress (Escobar-Sierra & Lampert, 2024; Pan et al., 2024).

During the warm season, stations PHE and AID exhibited greater impacts of salinization, with pathway enrichment influenced by higher electrical conductivities and chloride concentrations. The enrichment of ion transport pathways and key components of the transportome, particularly in gill tissues, aligns with consistent findings across various transcriptomic investigations that are discussed extensively in (Escobar-Sierra & Lampert, 2024) and (Escobar-Sierra et al., 2024). At PHE and AID, transport-related pathways were dominant, and pathways related to renal activity and hyperosmotic salinity stress were activated. These stations showed a physiological response similar to fish exposed to strong anthropogenic salinity pulses, including enriched osmoregulatory, immunological, and metabolism-related pathways (Escobar-Sierra et al., 2024). Key biomarkers such as *ABCA1*, *AQP3*, *KCNC2*,

WNK2, *WNK4*, *ANO6*, *ANO9*, and *KCNH* were enriched, indicating the activation of osmoregulatory and ion transport systems. WNKs (With-No-Lysine [K] kinases) are involved in ion transport, blood pressure regulation, and sodium-coupled chloride cotransport, playing a crucial role in regulating cell volume in response to osmotic stress (Kahle et al., 2010). KCNCs, like other voltage-gated Ca2+ channels, regulate ion and water transport, as well as Ca2+ signaling and entry in animal epithelia (J. Zheng & Trudeau, 2015). *AQP3*, part of the aquaporin gene family, facilitates water and solute transport in osmotic gradients and is differentially expressed in fish gill, kidney, and gut tissue in response to salinity stress across various species (Cutler & Cramb, 2002; Escobar-Sierra & Lampert, 2024; Giffard-Mena et al., 2007). *RAB21*, *RAB25*, and *RAB3A* are rab GTPases involved in vesicle transport and exocytosis. These small GTPases, including Ras and Rho, regulate ion homeostasis through direct interaction with ion channels, with their expression under salinity stress previously recorded in fish (Escobar-Sierra & Lampert, 2024; Pochynyuk et al., 2007). Notably, anoctamins *ANO6* and *ANO9*, and the ABC gene family member *ABCA1*, were also differentially expressed in the stressed stations during the cold season, underscoring their importance as candidate genes for assessing osmoregulation stress responses.

These findings reveal the critical role of the transportome in the adaptive response of freshwater fish to salinity changes, even at sublethal chloride concentrations. The study reveals the need for effective freshwater salinization control measures to conserve aquatic fauna in European streams. Salinization must be considered in any restoration efforts in the Emscher catchment before reintroducing endangered species like *Cottus rhenanus*. This pattern is likely applicable to other urban streams facing similar anthropogenic pressures. In summary, salinity stress significantly impacts the physiological responses of freshwater fish in the Emscher catchment, with key biomarkers indicating activation of osmoregulatory mechanisms regardless of

seasonal variations. This highlights the urgent need for addressing freshwater salinization in conservation and restoration efforts.

Immune system:

This study shows the significant effects of various stressors, such as salinity, water temperature, and oxidative stress, on the physiological responses of *Cottus rhenanus* in the urbanized Emscher catchment. Notably, the IL-17 and MAPK pathways are prominently activated at the AID and PHE stations during the warm season. Historically, research has concentrated on immune responses in primary and secondary lymphoid organs like the head, kidney, and spleen (Ewart et al., 2005; Overturf and LaPatra, 2006; Jørgensen et al., 2011). However, mucosal organs such as the gills, crucial for gas exchange and electrolyte balance, also play vital roles in pathogen defence (Evans et al., 1999; Koppang et al., 2015). Gills contain extensive mucosal surfaces and form the gill-associated lymphoid tissue, comprising various immune cells such as lymphocytes, macrophages, and antibody-secreting cells (Salinas, 2015).

Abiotic factors, including temperature fluctuations, crowding, salinity changes, and oxygen levels, profoundly influence fish immune systems. Previous studies have demonstrated that salinity stress, in particular, alters immune system processes with a notable involvement of the IL-17 and MAPK pathways (Escobar-Sierra et al., 2024). Furthermore, the IL-17 pathway have been found to be consistently activated in response to various stressors, such as ammonia, pathogens, heat, hypoxia, and salinity (Bai et al., 2022; X. Chen et al., 2021; Liang et al., 2022; Zhong et al., 2023). While the MAPK pathway is also frequently implicated in responses to stressors such as hypoxia and temperature changes (Lai et al., 2022; Song & McDowell, 2021; Tian et al., 2019; Y. Zhou et al., 2020). The IL-17 pathway, crucial for inflammatory responses,

is significantly enriched under stress conditions, underscoring its role in fish immune response. Similarly, the MAPK pathway, a conserved signal transduction route, responds to diverse extracellular stimuli and regulates processes such as proliferation, differentiation, development, stress response, survival, and apoptosis (Gehart et al., 2010). Our findings align with studies in teleost fish, showing MAPKs are involved in responses to stressors like heat, osmotic and oxidative stress, ionizing radiation, and inflammatory cytokines (W. Zheng et al., 2022).

The presence of several key genes relevant to the IL-17 and MAPK pathways at stressed stations during summer highlights the importance of these pathways in regulating stress responses to various abiotic and biotic stressors. Specifically, the shared genes between these pathways (*IL1B*, *TRAF6*, and *NFKB1*) illuminate their pivotal roles in mediating the body's response to environmental and stress-related challenges. *NFKB1*, a central regulator in the IL-17 pathway, orchestrates the transcriptional response to IL-17 signaling and contributes to the production of inflammatory mediators. This gene's interaction with the MAPK and JAK/STAT pathways is essential for fine-tuning immune responses and maintaining homeostasis. Previous studies have demonstrated that *NFKB1* is differentially expressed under various stress conditions in fish, highlighting its importance in managing immune responses and stress adaptation (Van Muilekom et al., 2023; Xie et al., 2024). *SOCS3*, another key gene in the IL-17 pathway, modulates inflammatory responses by providing negative feedback to cytokine signaling, preventing excessive inflammation and protecting tissues from damage (Alexander, 2002). *SOCS3*'s role in maintaining immune homeostasis and its differential expression under metabolic stress in fish species such as the arctic charr and Japanese flounder further supports its importance in stress management and tissue growth regulation (Deng et al., 2018; Jørgensen et al., 2013; S. Zhao et al., 2020).

IL-1β is a significant player in both the IL-17 and MAPK pathways. In the IL-17 pathway, IL-1β enhances Th17 cell differentiation and the expression of inflammatory genes, while in the MAPK pathway, it activates MAPKs to drive the production of inflammatory mediators. This dual role in regulating inflammation and stress responses highlight *IL-1β*'s importance in coordinating cellular reactions to environmental changes affection (Krasnov et al., 2020; Rebl et al., 2020; Syahputra et al., 2020). *TRAF6*, as an adaptor protein, is crucial for mediating inflammatory and immune responses in both pathways. In the IL-17 pathway, *TRAF6* facilitates NF-κB and MAPK activation, leading to pro-inflammatory gene expression. In the MAPK pathway, *TRAF6*'s role in MAPK activation influences inflammation, cell differentiation, and survival. The relevance of *TRAF6* in oxidative stress and its response to environmental factors such as hypoxia in fish emphasize its broad impact on stress responses and immune regulation (Z. Li et al., 2019; R. Wang et al., 2023).

In addition to the shared genes, the MAPK pathway components such as *JUNB*, *EGR1*, *ATF4*, and *SMAD4* further illustrate the pathway's complexity. *JUNB* and *EGR1* are crucial for regulating gene expression in response to extracellular signals, impacting inflammation, proliferation, and stress responses (Tiwari et al., 2013). *ATF4*'s role in managing oxidative stress and protein homeostasis highlights its importance in cellular stress adaptation, while *SMAD4*'s cross-talk with MAPK pathways underscores its significance in regulating responses to external stimuli (R. Wang et al., 2023; G. Zhang et al., 2016).

In summary, the interplay between the IL-17 and MAPK pathways, particularly through the shared genes *IL1B*, *TRAF6*, and *NFKB1*, demonstrates a strong immune response to multiple

stressors in more anthropogenic-influenced stations during the summer. These genes are important candidates for assessing stress in wild fish under multiple stressors, suggesting that targeting these pathways in stress response studies is crucial for monitoring the health of freshwater fish and their ecosystems. Future research should validate these findings across various species and ecosystems.

Wider implications and future directions:

Multiple stressors and physiological response:

This study identifies the physiological responses of fish to stressors in an urbanized multiple-stressor scenario in the wild. By relating the enrichment of various physiological pathways to increasing anthropogenic stressors such as decreasing dissolved oxygen, freshwater salinization, and temperature stress, candidate genes for rapid assessment of wild fish health were identified. Utilizing transcriptomics technologies allowed a holistic assessment of fish responses to multiple stressors in real-life scenarios, providing insights into the interactive effects on their overall physiological responses. Our findings align with the "metabolic compensation" strategy described by (Sokolova, 2013), where fish respond to stress by reallocating energy to defence mechanisms and maintenance, often at the expense of growth and reproduction and that Petitjean et al. (2019) consider is the response to single stressors. This strategy helps maintain homeostasis and survival but may transiently compromise the immune system. However, the response we describe is more in line with the pattern described by (Tort, 2011) regarding the response of fish to chronic stress, common in anthropogenically impacted environments, results in sustained energy reallocation to stress responses, which often translated in an altered immune function.

Interestingly, our results challenge the "metabolic conservation" strategy proposed by Petitjean et al. (2019), which suggests that under multiple stressors, high energy demands for maintenance may lead to a metabolic shutdown. This discrepancy may arise because previous observations were based on controlled lab conditions with fewer and more acute stressors. Our approach offers empirical insights in realistic ecological conditions, addressing the call for more studies to understand the physiological endpoints of individuals exposed to multiple stressors.

Future research should aim to establish a unifying conceptual framework for the physiological response to multiple stressors, similar to the Asymmetric Response Concept (ARC) proposed for ecosystem responses (Vos et al., 2023). This framework should consider the allostatic load or intensity of the stressor, gene expression changes, energy expenditure for homeostasis, and recovery trajectories after exposure to stressors. Efforts by Gandar et al., (2017) and Petitjean et al. (2019) have laid the groundwork, but new studies using omics tools provide a more holistic assessment. With the rapid advancement and affordability of high-throughput technologies, the comprehensive understanding of multiple stressor physiological responses is within reach and should be prioritized as a research direction in the field.

Transcriptomics in conservation of freshwater fish:

Assessing the overall health or physiological status of organisms has traditionally focused on model species under controlled conditions with few stressors. The advent of de novo transcriptomics now enables work with non-model species without published genomes. Advances in RNA-seq affordability and bioinformatics have expanded its applicability to

environmental sciences. As many reviews have stated, we are closer to accurately been able to assess the physiological status of organisms using transcriptomics with enormous implications to conservation (Connon et al., 2018; Jeffries et al., 2021; Semeniuk et al., 2022; Torson et al., 2020). Significant steps have demonstrated its applicability in unraveling the pathways and mechanisms of stress in wild fish (Escobar-Sierra et al., 2024; Escobar-Sierra & Lampert, 2024; Jeffrey et al., 2023; Komoroske et al., 2016). Specifically, we discerned the effects and their weight on the physiological response of sublethal stressors in a real-life scenario with multiple stressors interacting. The candidate genes identified in this study could be used for rapid assessment of anthropogenic stress in *Cottus rhenanus*, guiding conservation strategies for this endangered species. Rapid screening tools using candidate gene expression from transcriptomic research are being actively explored for assessing wild fish health (Chapman et al., 2021; Jeffries et al., 2021). Furthermore, our study identifies freshwater salinization, dissolved oxygen, and water temperature as highly influential stressors affecting the physiological response and immune system of *Cottus rhenanus*. For conservation of current populations in the Emscher catchment or planning new reintroductions in restored sites, these stressors must be carefully managed.

This study highlights the value of transcriptomics in assessing the health of non-model species in real-world scenarios. Future research should focus on refining rapid screening tools using candidate genes and exploring the physiological responses to multiple stressors in various species and environments. This approach will enhance conservation strategies and improve the management of endangered species under multiple anthropogenic stressors.

Conclusion:

Our transcriptomic study provides a comprehensive analysis of the physiological responses of *Cottus rhenanus* to multiple anthropogenic stressors in an urban river system. By leveraging high-throughput RNA sequencing and robust bioinformatics analysis, we identified significant variations in gene expression related to metabolism, oxidative stress, osmoregulation, transport, and immune responses, particularly in response to seasonal changes. The findings reveal a complex interplay between various stressors, such as high temperatures, salinity, low dissolved oxygen, and chemical pollutants, that collectively impact fish health and stress resilience. This study underscores the importance of understanding the holistic physiological responses of fish to multiple, concurrent stressors in their natural environment. Our identification of key biomarkers and enriched pathways offers valuable insights for monitoring fish health and informing conservation strategies. Future research should aim to integrate these transcriptomic insights into broader ecological assessments and management practices to mitigate the impact of urbanization on aquatic ecosystems and support the conservation of endangered species like *Cottus rhenanus*.

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Conclusive summary and future directions

1. Conclusive Summary

This thesis investigates the complex physiological responses of freshwater fish to salinity stress and multiple anthropogenic stressors, using a transcriptomic approach across three distinct studies. Each chapter contributes to a growing body of knowledge regarding how freshwater fish, ranging from the euryhaline *Gasterosteus aculeatus* to the invasive minnows (*Phoxinus septimaniae* × *Phoxinus dragarum*) and the sensitive *Cottus rhenanus*, cope with and adapt to changing environmental conditions, particularly in the context of freshwater salinization.

In the first study, *Gasterosteus aculeatus* was sampled in stations with subtle variations of salinity levels, even those below recognized toxic thresholds. The study revealed that salinity changes induce significant gene expression changes, particularly in genes associated with osmoregulation. This finding suggests that sublethal chloride concentrations can have a profound effect on fish physiology, even in species that are generally considered highly adaptable to salinity fluctuations. The transcriptomic analysis highlighted the critical role of the transportome (a suite of genes involved in ion transport and homeostasis) in mediating the adaptive response to salinity. The identification of key genes involved in these pathways provides a foundation for developing molecular biomarkers that can be used to assess the impact of sublethal salinity stress in field populations. This research underscores the sensitivity of transcriptomic approaches in detecting early physiological changes (sub-lethal) before they manifest in more overt, potentially irreversible, health impacts (Connon et al., 2018; Jeffries et al., 2021; Komoroske et al., 2016).

The second study delves into the molecular responses of the invasive hybrid minnows (*Phoxinus septimaniae* × *Phoxinus dragarum*) in the Llobregat River, an environment heavily influenced by anthropogenic activities, including pollution and freshwater salinization. The findings reveal that despite the multiple stressors present, the genomic responses in these fish were primarily driven by salinity. The study identified tissue-specific responses, particularly in the brain, where pathways related to stress, reproduction, growth, immune responses, methylation, and neurological development were significantly impacted. The brain's heightened sensitivity to salinity stress highlights the potential long-term effects on cognitive functions and behaviour, which could have significant ecological consequences, especially for invasive species that may have an advantage under changing environmental conditions. The

study suggests that the ability of these minnows to thrive in polluted and saline environments could be attributed to their robust osmoregulatory and stress response mechanisms, providing insights into the physiological traits that facilitate their invasiveness. The application of transcriptomics in this context proves valuable for understanding the underlying mechanisms of invasion success and for identifying potential molecular targets for controlling invasive species in affected ecosystems (Davidson et al., 2011; Gomes-Silva et al., 2020; Maceda-Veiga et al., 2010).

The third study expands the scope of investigation by exploring the responses of *Cottus rhenanus* to multiple anthropogenic stressors, including freshwater salinization, low dissolved oxygen, and elevated water temperatures, in an urban river system. This study underscores the complex interplay of stressors that fish experience in natural settings, particularly in urbanized areas where stressors often occur concurrently. The transcriptomic analysis revealed significant seasonal variations in gene expression related to key physiological processes such as metabolism, oxidative stress, osmoregulation, transport, and immune responses. Importantly, freshwater salinization emerged as a consistently influential stressor, significantly affecting the expression of genes involved in these critical pathways. This finding aligns with the results from the first two studies, reinforcing the conclusion that salinity stress is a pervasive and dominant factor affecting fish health, even in the presence of multiple other stressors. The research highlights the compounded effects of multiple stressors, which can exacerbate the physiological challenges faced by fish, leading to potential declines in population health and resilience (Sokolova, 2013; Tort, 2011). The study also emphasizes the utility of transcriptomics for monitoring the cumulative impacts of multiple stressors, providing a more nuanced understanding of how fish cope with the combined pressures of urbanization and environmental change

Drawing parallels across these three chapters, a consistent pattern emerges: salinity stress stands out as a critical factor influencing the physiological health of freshwater fish, regardless of the presence of other anthropogenic stressors. While the first two studies focused solely on salinity, revealing its profound impact even at sublethal levels, the third study broadened the analysis to include multiple stressors, yet still identified salinity as a key driver of physiological responses. This consistent finding across different species and environmental contexts underscores the importance of addressing freshwater salinization as a major ecological threat, particularly in regions where salinity levels are rising due to human activities such as agriculture, urban runoff, and industrial discharges.

The ecological implications of these findings are significant. Freshwater salinization not only affects individual fish health but also has broader consequences for ecosystem dynamics, including species interactions, community structure, and ecosystem functions. Salinity stress can alter predator-prey relationships, reproductive success, and the overall resilience of aquatic communities, potentially leading to shifts in species composition and the loss of biodiversity. The research presented in this thesis contributes to a deeper understanding of how freshwater ecosystems are impacted by salinity and provides valuable insights for the management and conservation of these vital habitats.

2. Future Directions

The findings of this thesis pave the way for future research that integrates transcriptomics and epigenetics to provide a more comprehensive understanding of how multiple stressors shape the physiological responses and adaptability of organisms in changing ecosystems. Traditional methods for assessing stressors' effects on organisms, such as endpoint mortality and lab-based experiments, often fail to capture the full complexity of natural environments, where multiple stressors interact simultaneously. Advances in molecular techniques, particularly transcriptomics and epigenetics, offer deeper insights into the physiological responses of organisms by evaluating changes in gene expression and epigenetic modifications.

Transcriptomics has proven effective in identifying the molecular pathways affected by multiple stressors and specific genes that respond to environmental challenges. For instance, studies have shown that transcriptomic approaches can reveal the activation of stress-related pathways in response to sublethal levels of pollutants, providing early-warning indicators of ecosystem health (Jeffries et al., 2021; Semeniuk et al., 2022). Future research should focus on refining these transcriptomic tools to assess the health of non-model species in real-world scenarios. This includes developing rapid screening methods using candidate gene expression to monitor the physiological status of wild fish populations exposed to multiple stressors.

Epigenetics, particularly DNA methylation, offers an additional dimension of insight into how organisms respond to environmental stressors. DNA methylation can regulate gene expression without altering the underlying nucleotide sequence, providing a mechanism for phenotypic plasticity and rapid adaptation. These epigenetic changes can be stable and heritable, making them valuable biomarkers for environmental stress (Best et al., 2018; Herrel et al., 2020). Integrating epigenetic analysis with transcriptomics will allow researchers to explore the interactions between genes, environment, and phenotypes more comprehensively. Such

integrative approaches are increasingly used in ecological and evolutionary research to study how organisms adapt to changing environments and to identify the long-term effects of multiple stressors on organismal physiology (Lamka et al., 2022).

Specifically, future studies should aim to establish a unifying conceptual framework for understanding the physiological response of fish to multiple stressors, similar to the Asymmetric Response Concept (ARC) proposed for ecosystem responses (Vos et al., 2023). This framework should account for the intensity and duration of stressors, gene expression changes, energy expenditure for homeostasis, and recovery trajectories after exposure to stressors. By incorporating both transcriptomic and epigenetic data, researchers can gain a more holistic understanding of how fish and other aquatic organisms cope with the complex stressor interactions that characterize urbanized and industrialized environments.

Moreover, the application of these molecular techniques should be expanded to other non-model species and ecosystems. Studies in species such as *Cottus* spp., which are particularly sensitive to anthropogenic stressors, can provide critical insights into the broader impacts of environmental change on freshwater biodiversity (Markert et al., 2024). In addition, these approaches can be applied to monitor the success of conservation efforts, such as the reintroduction of endangered species into restored habitats, by assessing the physiological and epigenetic responses of individuals to their new environments.

In conclusion, the integration of transcriptomics and epigenetics represents a promising direction for future research in conservation biology. By advancing our understanding of how environmental stressors influence the molecular and physiological responses of organisms, these approaches will enhance our ability to protect and manage freshwater ecosystems in the face of global environmental change.

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Sub-publications and records of achievement

Chapter 1: Escobar-Sierra, C., & Lampert, K. P. (2024). Field application of de novo transcriptomic analysis to evaluate the effects of sublethal freshwater salinization on *Gasterosteus aculeatus* in urban streams. Plos one, 19(3), e0298213. Original paper. Published. https://doi.org/10.1371/journal.pone.0298213

Contribution: Writing – original draft, writing – review & editing, fieldwork, visualization, investigation, formal analysis, data curation, conceptualization.

Chapter 2: Escobar-Sierra, C., Cañedo-Argüelles, M., Vinyoles, D., & Lampert, K. P. (2024). Unraveling the molecular mechanisms of fish physiological response to freshwater salinization: A comparative multi-tissue transcriptomic study in a river polluted by potash mining. *Environmental Pollution*, 124400. Original paper. Published. https://doi.org/10.1016/j.envpol.2024.124400

Contribution: Writing – original draft, fieldwork, writing – review & editing, visualization, investigation, formal analysis, data curation, conceptualization.

Chapter3: Escobar-Sierra, C., & Lampert, K. P. (2024). Navigating the Urban River: Transcriptomic Responses of Freshwater Fish to Multiple Anthropogenic Stressors. Original paper. Preprint. https://doi.org/10.1101/2024.08.19.608252

Contribution: Writing – original draft, fieldwork, writing – review & editing, visualization, investigation, formal analysis, data curation, conceptualization.

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Lastly, and most importantly, I dedicate this work to my wife, Stephanie Carvajal Acevedo, my life partner, best friend, and love. Thank you for your unwavering support and love throughout this journey.

Erklärung gemäß § 7 Absatz 8 der Promotionsordnung

Erklärung zur Dissertation
gemäß der Promotionsordnung vom 12. März 2020

Diese Erklärung muss in der Dissertation enthalten sein.
(***This version must be included in the doctoral thesis***)

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

1. Escobar-Sierra, C., Cañedo-Argüelles, M., Vinyoles, D., & Lampert, K. P. (2024). Unraveling the molecular mechanisms of fish physiological response to freshwater salinization: A comparative multi-tissue transcriptomic study in a river polluted by potash mining. Environmental Pollution, 124400. Original paper. Published. https://doi.org/10.1016/j.envpol.2024.124400
2.Escobar-Sierra, C., & Lampert, K. P. (2024). Field application of de novo transcriptomic analysis to evaluate the effects of sublethal freshwater salinization on Gasterosteus aculeatus in urban streams. Plos one, 19(3), e0298213. Original paper. Published. https://doi.org/10.1371/journal.pone.0298213

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Curriculum vitae

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WORK EXPERIENCE

01/04/2021 – 25/10/2024 Köln, Germany
RESEARCHER AND PH.D. CANDIDATE UNIVERSITY OF COLOGNE

Understanding the effects of multiple anthropogenic stressors effects on freshwater fish physiology. Defence Completed on 25 Oct 2024.

01/09/2019 – 01/09/2020 Medellin, Colombia
AQUATIC ECOLOGY CONSULTANT HIDROBIOLÓGICA S.A.S

Prepared environmental impact assessment reports for aquatic ecosystems affected by infrastructure projects. Conducted fish sampling using techniques such as angling, electrofishing, and fish traps.

01/11/2019 – 01/11/2020 Plasencia, Belize
AQUACULTURE CONSULTANT B-BLUE

Designed a pilot research project for giant grouper aquaculture as an alternative livelihood for the local community and to establish species reintroduction. Developed guidelines for hatchery establishment and species cultivation.

01/02/2019 – 01/03/2019 Bogota, Colombia
AQUATIC ECOLOGY CONSULTANT REFORESTADORA DE LA COSTA S.A.S

Coordinated fish rescue operations during a drought contingency at a hydroelectric project, leading a team of 10 assistants to rescue stranded fish.

01/09/2015 – 01/08/2016 Medellin, Colombia
BIOLOGICAL COLLECTIONS MANAGER NATURAL HISTORY MUSEUM, UNIVERSIDAD DE ANTIOQUIA

Led a ten-person team managing the museum's biological collections. Contributed to science communication and served on the editorial board of the museum journal.

01/03/2014 – 01/03/2015 Medellin, Colombia
AQUACULTURE CONSULTANT ECOTEC INVESTMENTS S.A.S

Developed a project for intensive Tilapia cage culture in a tropical reservoir. Conducted research, infrastructure development, budget design, and viability assessment.

01/01/2014 – 01/01/2015 Bogota, Colombia
AQUATIC ECOLOGY CONSULTANT FUNDACIÓN OMACHA

Coordinated the assessment and monitoring of artisanal and recreational fisheries in three freshwater reservoirs in the Colombian Andes.

01/06/2013 – 01/11/2013 Medellin, Colombia
LECTURER IN RIVER ECOLOGY UNIVERSIDAD DE ANTIOQUIA

I taught an elective course on river ecology for third-year biology undergraduates, emphasizing the application of science to conservation and natural resource management.

Curriculum vitae

EDUCATION AND TRAINING

21/04/2021 – 25/10/2024 Cologne, Germany
PHD BIOLOGICAL SCIENCES University of Cologne

Successfully defended the thesis on the 25th OCT 2024.

Website https://zoologie.uni-koeln.de/arbeitsgruppen/ag-lampert/inhalt/mitarbeiterinnen/camilo-escobar-sierra |

Final grade Magna Cum Laude | **Level in EQF** EQF level 8 |

Thesis Physiological responses of freshwater fish to multiple stressors in urban rivers: a transcriptomic approach

12/09/2016 – 30/07/2018 Oban, Scotland, United Kingdom
JOINT MASTER DEGREE IN AQUACULTURE, ENVIRONMENT AND SOCIETY (ACES) University of the Highlands and Islands, University of Crete and Nantes Université

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01/07/2006 – 25/10/2013 Medellin, Colombia
BSC HONS BIOLOGY Universidad de Antioquia

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Level in EQF EQF level 6

LANGUAGE SKILLS

Mother tongue(s): **SPANISH**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
ENGLISH	C2	C2	C1	C1	C2
GERMAN	B2	B1	A2	A2	A2

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user

PUBLICATIONS

2024
Navigating the Urban River: Transcriptomic Responses of Freshwater Fish to Multiple Anthropogenic Stressors

Escobar-Sierra, C., & Lampert, K. P. bioRxiv 2024.08.19.608252. Original paper. Submitted.

2024
Global thermal tolerance of freshwater invertebrates and fish

Helena S. Bayat, Fengzhi He, Graciela M. Madariaga, Camilo **Escobar-Sierra**, Sebastian Prati, Jonathan F. Jupke, Kristin Peters, Xing Chen, Jurg W. Spaak, Alessandro Manfrin, Noel P.D. Juvigny-Khenafou, Ralf B. Schäfer. BioRxiv 2024.07.08.602306. Original paper. Submitted.

2024
Unraveling the molecular mechanisms of fish physiological response to freshwater salinization: A comparative multi-tissue transcriptomic study in a river polluted by potash mining

Escobar-Sierra, C., Cañedo-Argüelles, M., Vinyoles, D., & Lampert, K. P. Environmental Pollution, 124400. Original paper. Published.

2024
Field application of de novo transcriptomic analysis to evaluate the effects of sublethal freshwater salinization on Gasterosteus aculeatus in urban streams

Escobar-Sierra, C., & Lampert, K. P. Plos one, 19(3), e0298213. Original paper. Published.

2024
Solving the Puzzle of Ecosystem Recovery

Bayat HS, Enß J, **Escobar-Sierra C,** Gillmann SM, Khaliq S, Kuppels A, Madariaga GM, Peters K, Schlenker A, Hering D and Vos M. Front. Young Minds. 12:1302974. Original paper. Published.

2023
The Asymmetric Response Concept explains ecological consequences of multiple stressor exposure and release

Matthijs Vos, Daniel Hering, Mark O. Gessner, Florian Leese, Ralf B. Schäfer, Ralph Tollrian, Jens Boenigk, Peter Haase, Rainer Meckenstock, Daria Baikova, Helena Bayat, Arne Beermann, Daniela Beisser, Bánk Beszteri, Sebastian Birk, Lisa Boden, Verena Brauer, Mario Brauns, Dominik Buchner, Andrea Burfeid-Castellanos, Gwendoline David, Aman Deep, Annemie Doliwa, Micah Dunthorn, Julian Enß, Camilo **Escobar-Sierra**, Christian K. Feld, Nicola Fohrer, Daniel Grabner, Una Hadziomerovic, Sonja C. Jähnig, Maik Jochmann, Shaista Khaliq, Jens Kiesel, Annabel Kuppels, Kathrin P. Lampert, T.T. Yen Le, Armin W. Lorenz, Graciela Medina Madariaga, Benjamin Meyer, Jelena H. Pantel, Iris Madge Pimentel, Ntambwe Serge Mayombo, Hong Hanh Nguyen, Kristin Peters, Svenja M. Pfeifer, Sebastian Prati, Alexander J. Probst, Dominik Reiner, Peter Rolauffs, Alexandra Schlenker, Torsten C. Schmidt, Manan Shah, Guido Sieber, Tom Lennard Stach, Ann-Kathrin Tielke, Anna-Maria Vermiert, Martina Weiss, Markus Weitere, & Bernd Sures. *Science of the Total Environment, 872, 162196.* Original paper. Published.

2022
Metabolomics Unravels Grazing Interactions under Nutrient Enrichment from Aquaculture

Escobar-Sierra C, de Kock W, Hasler-Sheetal H, Holmer M, Chatzigeorgiou G, Tsapakis M, & Apostolaki ET. Diversity, 15(1), 31. Original paper. Published.

2021
An updated reef fish checklist of the southernmost Caribbean reef system, with comments on the lionfish invasion

Escobar-Sierra C, Márquez Velásquez V, Menezes R, Souza Rosa R, & Loaiza-Santana A. Biota colombiana, 22(2), 70-87. Original paper. Published.

2014
Diadromy as evolutionary convergence in fish, shrimp and snails in the peri-continental basins of Colombia

Blanco JF, Carvajal JD, **Escobar-Sierra C**, Jiménez LF, Lasso CA & Sánchez-Duarte P. . Instituto de Investigación de los Recursos Biológicos Alexander von Humboldt (IAvH). Bogotá, D. C., Colombia. Digital ISBN: 978-958-8889-26-9. Book chapter. Published.

2014
San Jacinto Mountain range (case study). Serie Editorial Recursos Hidrobiológicos y Pesqueros Continentales de Colombia.

Blanco JF & **Escobar-Sierra C**. Instituto de Investigación de los Recursos Biológicos Alexander von Humboldt (IAvH). Bogotá, D. C., Colombia. ISBN Digital: 978-958-8889-26-9. Book chapter. Published.

2014
Gorgona, Baudó and Darién (Biogeographic Chocó, Colombia): model ecoregions for coastal stream ecology studies

Blanco JF, **Escobar-Sierra C** & Carvajal-Quintero JD. Revista de Biología Tropical, 62(1), 43-64. Original paper. Published.

CONFERENCES & SEMINARS

05/05/2024 – 09/05/2024 Seville, Spain
SETAC 34th European Annual Meeting

- Poster/presentation: Differential Gene Expression of Freshwater Macroinvertebrates Exposed to Micropollutant Mixtures across the River Holtemme (Germany).
- Poster: Unravelling the Molecular Mechanisms of Fish Salinity Adaptation in the Face of Severe Osmotic Stress: A Comparative Multi-Tissue Transcriptomic Study in the Llobregat River, Barcelona, Spain.

19/06/2023 – 23/06/2023 Newcastle, UK
13th Symposium for European Freshwater Sciences

- Presentation: Comparative transcriptomics reveals molecular mechanisms of European fish salinity adaptation under multiple stressors.

30/04/2023 – 04/05/2023 Dublin, Ireland
SETAC 33rd European Annual Meeting

- Poster: The Use of Comparative Transcriptomics Field Studies to Assess the Effects of Multiple Stressors on Fish.

11/10/2022 – 14/10/2022 Berlin, Germany
13th YOUMARES - Conference for young marine researchers

- Session Chair and Presenter: Omics application in aquatic science research.

07/08/2022 – 10/08/2022 Berlin, Germany
36th Congress of the International Society of Limnology

- Poster: Transcriptome response of Gasterosteus aculeatus in natural habitats affected by multiple anthropogenic stressors.

20/11/2018 – 23/11/2018 Medellín, Colombia
1st International Conference in Marine Science

- Presentation: Mediterranean aquaculture impact on subtidal macroalgae community: a case study from the Aegean Sea (Greece).

23/06/2013 – 27/06/2013 San José, Costa Rica
50th Anniversary Meeting ATBC and OTS

- Poster: Factors influencing longitudinal patterns of stream fishes in a coastal semi-arid landscape (Caribbean dry forest, Colombia).

27/05/2013 – 31/05/2013 Bogotá, Colombia
12th Colombian Ichthyological Congress and 3rd South American Ichthyologist Meeting

- Presentation: Fishes in a neotropical semi-arid landscape: factors influencing their species composition patterns.

RESEARCH STAYS

03/2024 – 04/2024
Research Stay to Establish Collaboration on Fish Stress Epigenetics

Collaborated with Dr. Janan Gawra and Dr. Laia Navarro-Martín of IDAEA-CSIC (Barcelona) to implement and train on the EPIGBS2 protocol for assessing DNA methylation, focusing on adaptation in freshwater fish under multiple stressors in urban and agricultural streams.

INVITED TALKS

10/2024 – 10/2024
From genes to ecosystems: Omics approaches for decoding aquatic organism responses to environmental change

Invited talk at the Institute of Ecotoxicology seminar, RWTH Aachen, Germany

04/2024 – 04/2024
Multiple stressor effects on sculpins (Cottus sp.), sticklebacks (G. aculeatus) and related top-down effects on riverine food-webs

Invited talk at the group of Environmental Toxicology seminar from Dr Laia Navarro, IDAEA-CSIC, Barcelona

PEER REVIEWER FOR SCIENTIFIC JOURNALS

Aquaculture Reports

Aquaculture Research

HONOURS AND AWARDS

01/04/2021
Fully funded Phd position – University of Cologne

01/09/2016
Full Scholarship Erasmus Mundus+ for the Joint Master Degree in AquaCulture, Environment, and Society – European Eunion

01/10/2012
Best wildlife photography award for the cover of the Hipotesis journal – Hipotesis journal

01/06/2012
University of Florida Scholarship to attend the Field course in research design – University of Florida

01/02/2012
Idea Wild 1000 USD equipment and supplies grant for conservation projects – Idea Wild

01/01/2009
Assistantship for academically outstanding students – Universidad de Antioquia

- **NETWORKS & MEMBERSHIPS**

  Society of Environmental Toxicology and Chemistry (SETAC)

  Centre of Water and Environmental Research (ZWU)

- **DIGITAL SKILLS**

  Galaxyproject.org | R user | QGIS: basic level | Bionformatics | Image Editing | Computer proficiency

- **HOBBIES AND INTERESTS**

  PADI Open Water Diver Certificate, PADI Deep Dive Skill, Free Diving

  Wildlife and Underwater Photography

  Hiking and Trekking